THE ACTION OF RICIN A CHAIN ON EUKARYOTIC RIBOSOMES

Rupert W. Osborn, BA (Hons.) Cantab

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Department of Biological Sciences

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To Annie

'We often discover what will do, by finding
out what will not do; and probably he who
never made a mistake never made a discovery.'

Samuel Smiles,1863.

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DECLARATION

All the results presented in this thesis were obtained by the author. Where appropriate, the sources of information and materials have been specifically acknowledged in the text. Some of the results presented in chapters 3 and 4 have been published in May et al. (1989) and Osborn, R.W. and Hartley, M.R., Eur. J. Biochem. (1990) **193**: 401-407. None of the work contained in this thesis has been used for the previous application for a degree.

R.W.Osborn

SUMMARY

The action of ricin A chain on eukaryotic ribosomes was investigated from a number of different angles. It was shown that ricin A chain modified the rRNA from the 60S subunit of a number of eukaryotic ribosomes, including plant ribosomes, and that the site of action was at the same position in a highly conserved sequence which had previously been identified as the site of action in rat liver 28S rRNA.

Investigations into the partial reactions of protein synthesis inhibited in ricin A chain-treated ribosomes showed that both initiation and elongation were inhibited, contradicting the assumption based on previous work that ricin A chain inhibited just the elongation cycle. In a rabbit reticulocyte lysate it was found that whilst elongation was severely inhibited by ricin A chain, the rate of initiation was also reduced approximately six-fold relative to that seen with an inihibitor of just elongation. Furthermore, experiments carried out to investigate the inhibition of elongation showed that this was most likely due to the inhibition of the elongation factor 2 catalysed step.

Using an assay which allowed the N-glycosidase activity of ricin A chain to be measured directly it was possible to show that ribosomes from a number of different sources varied markedly in their sensitivity to ricin A chain. Wheat germ ribosomes were shown to be in the order of 1000 times less sensitive to modification than those from either yeast or rabbit reticulocytes. However, this difference does not seem to be a reflection of the differential affinity of ricin A chain for the various substrates but rather a consequence of the ability of the toxin to modify the rRNA once it has bound. This is because kinetic experiments showed that the Km for the reaction on wheat germ ribosomes was similar to that which had been published for the action of ricin A chain on rat liver or rabbit ribosomes. The Kcat, however, was approximately 3 orders of magnitude smaller. A similar picture was seen with the type 1 RIP dianthin 32.

It was shown directly that elongation factor 2 bound irreversibly to the ribosome could protect the ribosome from the action of ricin A chain and that in a wheat germ lysate this ability to compete out ricin A chain seemed to be a property of just this supernatant protein. This protection is reflected in the finding that the elongation factor 2 content of purified ribosomes determines their sensitivity to depurination by ricin A chain and that the removal of this protein with high salt sensitises the ribosomes to modification.

ABBREVIATIONS

A_{254nm}	absorbance at 254 nm
ADP	adenosine diphosphate
ATP,dATP	adenosine triphosphate, deoxyadenosine triphosphate
Arg	arginine
Asn	asparagine
b	base
bp	base pair
BSA	bovine serum albumin
cDNA	complementary DNA
Ci	curie
cpm	counts per minute
CTAB	hexadecyltrimethylammonium bromide
CTP,dCTP	cytidine triphosphate, deoxycytidine triphosphate
Da	dalton
DNA	deoxyribonucleic acid
dpm	disintegrations per minute
DTT	dithiothreitol
EDTA	ethylenediaminetetra-acetic acid
eEF	eucaryotic elongation factor
eIF	eucaryotic initiation factor
Glu	glutamic acid
GMPPCP	guanylyl(methylene) diphosphate
GTP,dGTP	guanosine triphosphate, deoxyguanosine triphosphate
Hepes	N-2-hydroxyethylpiperazine-N-2-ethane sulphonic acid
K_{cat}	turnover number or rate

K_m	Michaelis-Menton constant
l	litre
LSU-rRNA	large ribosomal subunit rRNA
M	molar
met	methionine
Met-tRNAi	initiator methionyl tRNA
min	minute
ml	millilitre
mM	millimolar
mRNA	messenger RNA
NAD	nicotinamide adenine dinucleotide
ng	nanogram
PAGE	polyacrylamide gel electrophoresis
PMSF	phenylmethyl sulfonyl flouride
poly (U)	poly uridylic acid
RIP	ribosome inactivating protein
RNA	ribonucleic acid
rpm	revolutions per minute
rRNA	ribosomal RNA
S	Svedberg unit (1×10^{-13} sec.)
[S]	concentration of substrate
SDS	sodium dodecyl sulphate
SSU-rRNA	small ribosomal subunit RNA
TCA	trichloroacetic acid
TEMED	N,N,N',N'-tetramethylethylenediamine
TLC	thin layer chromotography
Tris	trishydroxylaminomethane

tRNA	transfer RNA
Trp	tryptophan
dTTP	deoxythymidine triphosphate
U	units
µg	microgram
µl	microlitre
v	velocity
val	valine
V_{max}	maximum velocity
v/v	volume per volume
w/v	weight per volume

CHAPTER 1.

INTRODUCTION.

SECTION 1.1 HISTORICAL BACKGROUND.

Since ancient times extracts from certain plants have been exploited for their extreme toxicity and used for medicinal and criminal purposes alike. At the end of the last century Stillmark (1887) identified a protein which he concluded was the cause of the well recognised toxicity of extracts from the seeds of the castor oil plant Ricinus communis. He demonstrated that this protein was a haemagglutinin which he called ricin. It was not until the application of increasingly more sophisticated protein purification techniques that this was shown to be an oversimplification. The active component that Stillmark identified was found to be an equal mixture of two closely related proteins (Olsnes and Pihl, 1973). One of these proteins, Ricinus communis agglutinin (RCA), is a strong agglutinin but relatively non toxic to intact cells while the other is a potent cytotoxin but a weak haemagglutinin (Olsnes et al., 1974). It is this second protein that is still refered to as ricin.

Ricin is now classed as a ribosome inactivating protein which catalytically inactivates eukaryotic ribosomes. The considerable interest in this protein over the years is a reflection of its extreme potency and more recently a consequence of its use in the construction of immunotoxins.

SECTION 1.2 RIBOSOME INACTIVATING PROTEINS.

1.2.1 General Characteristics.

Ricin is one of a group of proteins which are structurally and functionally similar and which inhibit protein synthesis in eukaryotic *in vitro* translation systems (Jimenez and Vazquez, 1985, Olsnes and Pihl, 1982, Stirpe and Barbieri, 1986). The majority of these proteins have been isolated from plants and it has been suggested that all plants might contain related proteins which have evolved from a common ancestral gene (Ready *et al.*, 1984). The term ribosome inactivating protein (RIP) was proposed originally to classify these plant proteins into one group. The criteria for a protein to be classed as a RIP was that it must catalytically inactivate the 60S subunit of eukaryotic ribosomes rendering them incapable of binding elongation factor 2 (eEF-2) (Stirpe, 1982). Proteins which share this general property had also been found in fungi (Fernandez-Puentes and Vazquez, 1977) and bacteria (Reisbig *et al.*, 1981) but because the term RIP was coined before the mode of action of the plant proteins was known it was not clear exactly how related these other toxins were to the plant RIPs. It has now been shown that these bacterial proteins are similar in structure and function to the plant RIPs (Endo *et al.*, 1988d) and so can be included in this general class. The fungal cytotoxins, although different to the other RIPs in their activity, do still meet the criteria proposed by Stirpe to define an RIP and so should also be included.

1.2.2 Plant Ribosome Inactivating Proteins.

To date ribosome inactivating proteins have been found in at least 15 plant families with the strongest inhibitory activity found in plants belonging to the families Caryophyllaceae and Euphorbiaceae (Jimenez and Vazquez, 1985, Stirpe and Barbieri, 1986, Merino et al., 1990). Plant RIPs are broadly classified into two groups on the basis of their structure (Stirpe, 1982, Stirpe and Barbieri, 1986). Type 1 RIPs exist as single chain polypeptides of Mr approximately 30,000 and are usually glycosylated. They are characterised by being relatively non toxic to intact cells with the exception of macrophages. Type 2 RIPs are heterodimeric glycoproteins possessing a catalytic A chain and a cell surface-binding B chain, held together by a single disulphide bond. The A chains resemble the type 1 RIPs in their structure and function while the B chains are galactose binding lectins. Consequently these proteins are potent cytotoxins, binding to cells through carbohydrate moities of glycolipids and glycoproteins that contain terminal galactose residues and subsequently being internalised where the enzymatic A chains can inactivate ribosomes (Olsnes and Pihl, 1982). Some of the better characterised plant RIPs are listed in Table 1.2.1.

In plants containing type 2 RIPs there occurs related four subunit proteins. These have essentially the same structure as the two subunit toxins but complexes of subunits of the same type are linked by weak interactions, and therefore tetramers are formed having two A and two B subunits. The tetramers are strong agglutinins but are noncytotoxic. From peptide fingerprinting (Nicolson et

PROTEIN	SOURCE	REFERENCE
TYPE 1		
Barley inhibitor	*Hordeum vulgare*	Reisbig and Bruland (1983)
Dianthin 30,32	*Dianthus caryophyllus*	Stirpe *et al*. (1981)
PAP, PAP-II, PAP-S	*Phytolacca dodecandra*	Ready *et al*. (1984)
Gelonin	*Gelonium multiflorum*	Stirpe *et al*. (1980)
Saporin	*Saponaria officinalis*	Stirpe *et al*. (1983)
Tritin	*Triticum aestivum*	Roberts and Stewart (1979)
TYPE 2		
Ricin	*Ricinus communis*	Olsnes and Pihl (1973)
Abrin	*Abrus precatorius*	Olsnes *et al*. (1975a)
Modeccin	*Modecca digitata*	Gasperi-Campani *et al*. (1978)
Viscumin	*Viscum album*	Ziska *et al*. (1978)

Table 1.2.1 Examples of Plant Ribosome Inactivating Proteins.

al., 1974) and immunology studies (Saltvedt, 1976) it was suggested that the A and B subunits from ricin and the corresponding agglutinin, RCA, were similar in primary structure. Cloning of the primary sequence for both proteins has shown this to be the case (Roberts et al., 1985); the A chains are 93% homologous and the B chains are 84% homologous.

The location of RIPs within most plants is tissue specific with the specificity varying from species to species. RIPs have been isolated from seeds, roots, stems and leaves, and they vary in concentration form a few ug to several hundred mg per 100g tissue. Plants can contain more than one type of RIP. For example the leaves of Dianthus caryophyllus contain two type 1 RIPs, dianthin 30 and 32 (Stirpe et al., 1981) and Phytolacca dodecandra contains a seed specific RIP,pokeweed antiviral protein S (PAP-S), in addition to two leaf forms (Irvin, 1975). Dianthin 32 is found only in the leaves of the carnation plant whereas dianthin 30 is also present in the roots and seeds (Reisbig and Bruland, 1983a). In Phytolacca the two leaf RIPs show a temporal shift in their expression. One form (PAP or PAP-1) is predominant in spring leaves while PAP-2 is the predominant form in summer leaves (Irvin et al., 1980).

1.2.3 Anti-viral Activity.

Plant RIPs are potent antiviral agents. The ability of these proteins to inhibit the transmission of viruses was first definitively shown with PAP (Tomlinson et al., 1974) and accounted

for the earlier observations that extracts from a number of plants had an antiviral effect (Wyatt and Shephard, 1969). PAP inhibited the transmission of plant viruses and reduced the multiplication of mammalian viruses in infected cells (Tomlinson et al., 1974, Foa-Tomasi et al., 1982). Other type 1 RIPs and the A chains of type 2 RIPs also share this antiviral activity against the transmission of plant (Stevens et al., 1981, Ikeda et al., 1987) and mammalian viruses (Foa-Tomasi et al., 1982, Fernandez-Puentes and Carrasco, 1980).

Although the mechanism of RIP-mediated inhibition of virus replication has not been studied in detail it is not a result of the direct interaction of the RIP with the virus (Tomlinson et al., 1974) but rather the result of the inhibition of protein synthesis in infected cells (Owens et al., 1973, Foa-Tomasi et al., 1982). It has been suggested that viral infection permeabilises the cells and allows entry of proteins which would otherwise be unable to cross the plasma membrane (Fernandez-Puentes and Carrasco, 1980). This would explain the inhibition of protein synthesis since it would result in entry of the type 1 RIPs like PAP into the cytoplasm. Recently one study has addressed the molecular biology of virus inhibition. McGrath et al. (1989) reported that trichosanthin, a type 1 RIP from the root tubers of Trichosanthes kirilowii, inhibits HIV replication in acutely and chronically infected cells by specifically inhibiting viral RNA and protein synthesis without affecting host cell RNA synthesis. However the basis of this selectivity is not known.

Despite the well documented ability of RIPs to inhibit virus replication the question of whether this is the role of these

proteins in the plant remains an enigma. In the case of PAP, Ready et al. (1986) showed that the protein was exclusively located within the cell wall matrix. The authors hypothesised that the RIP could be released into the cytoplasm during insect, nematode or fungal vector penetration, thereby inhibiting protein synthesis and preventing replication of any viruses introduced by the vector. However as a general mechanism this is inconsistent with the observations that the ribosomes of many plants are resistant to their endogenous RIPs (Battelli et al., 1984) and plant extracts are unable to inhibit viral replication in plants from which they are derived (Hansen, 1989). It therefore remains to be conclusively shown why these proteins are apparently ubiquitous in plants.

1.2.4 Bacterial Ribosome Inactivating Proteins.

Cytotoxins which catalytically inactivate the 60S subunits of eukaryotic ribosomes are also found in Shigella dysenteria type 1 (Reisbig et al., 1981) and certain strains of E.coli (Igarashi et al., 1987). The enterohaemorrhagic E.coli toxins were identified by their cytotoxic activity against Vero cells (Konowalchuk et al., 1977) and given the name vero toxins. Subsequently at least two immunologically distinct vero toxins were identified, VT1 and VT2 (Scotland et al., 1985). The former has been shown to be biologically identical to Shiga toxin from Shigella dysenteria (Noda et al., 1987). Due to this similarity in activity the vero toxins are now refered to as Shiga-like toxins, SLT 1 and SLT 2.

In contrast to the cytotoxic plant RIPs the bacterial RIPs

contain a single A chain and multiple copies of a B chain which bind to receptors on the cell-surface (Jacewicz et al., 1986). However the nucleotide sequence of the Shiga-like toxin genes of E.coli has been determined and the A subunit was found to highly homologous to ricin A chain (Calderwood et al., 1987). Subsequently it has been shown that the bacterial RIPs have exactly the same catalytic specificity as ricin A chain (Endo et al., 1988d). This close homology in divergent organisms suggests that the two groups of toxins evolved from a common ancestral gene and it is interesting to note that the bacterial RIPs resemble the plant RIPs and not the fungal toxins described next.

1.2.5 α-sarcin.

α-sarcin is one of three cytotoxic proteins produced by different strains of the mold Aspergillus. These proteins restrictocin, mitogillin and α-sarcin show a high degree of homology with each other (Lopez-otin et al., 1984) and have been shown to catalytically inactivate both eukaryotic and prokaryotic ribosomes (Fernandez-Puentes and Vazquez, 1977, Schindler and Davies, 1977). The complete nucleotide sequence of the cDNA for α-sarcin has been determined (Oka et al., 1990) and confirmed earlier analysis that it is a basic protein with a Mr of 16,987 (Sacco et al., 1983).

Inactivation of the 60S subunit of eukaryotic ribosomes results from the hydrolysis of a single phosphodiester bond near the 3' end of the large ribosomal subunit RNA (Schindler and Davies, 1977). In rat 28S rRNA the cleavage site is between G_{4325} and A_{4326}

(Endo and Wool, 1982, Chan et al., 1983a). As will be discussed in the following sections the activity of α-sarcin is different from the other RIPs described above. The action of α-sarcin results in cleavage of the rRNA backbone, whilst this is not the case with the plant and bacterial RIPs where depurination of the rRNA leaves the backbone intact. The fungal toxins described here are also completely different in their primary sequence and consequently their secondary structure. However the site of action of α-sarcin is only one base to the 3' of the site of action of ricin A chain and so the two proteins might be expected to be similar in the way they interact with the ribosome. The consequence of the action of these proteins on the activity of the ribosome and in particular the steps of protein synthesis which are inhibited might also be expected to be similar.

SECTION 1.3 RICIN-STRUCTURE AND FUNCTION.

1.3.1 Structure of the A and B Chains.

Ricin is expressed exclusively in the castor bean endosperm cells and rapidly degraded during seed germination (Roberts and Lord, 1981). The complete primary sequence of the A and B chains has been determined both directly by protein sequencing (Funatsu et al., 1979) and by DNA sequencing of cDNA clones (Lamb et al., 1985). The two chains are synthesised together in a precursor polypeptide (proricin) which is cleaved by an acid endopeptidase(s) within the protein bodies to generate the mature protein. Initial synthesis of the protein is by way of a preproprotein which is made in the

cytoplasm and cotranslationaly targetted to the endoplasmic reticulum where the signal sequence is cleaved allowing the protein to fold and the formation of disulphide bonds (reviewed in Lord et al., 1987). Preproricin contains a 35 amino acid leader sequence and a 12 amino acid linker peptide between the A and B chains (Halling et al., 1985). From consensus sequences of other signal sequences it is unlikely that the entire 35 amino acid leader sequence encodes for the signal sequence (Lamb et al., 1985). More probable is that the cleavage site lies within the 35 amino acids, and further processing would be required to reveal the mature A chain N-terminus. It is possible that this N-terminal processing is carried out by the same endopeptidase responsible for the cleavage of the linker peptide in the protein bodies (Lord et al., 1987).

Both the A and B chains of ricin contain two N-glycosylation sites (Lamb et al., 1985) although purified A chain is found in two forms, a 32,000 and a 34,000 Mr protein which represent glycosylation variants (Foxwell et al., 1985). Ricin A chain has been expressed in E.coli and shown to be soluble and have full biological activity comprable with the native plant purified A chain (O'Hare et al., 1987). Expression of recombinant B chain has proved more problematic because the expressed protein is insoluble and requires refolding for it to have lectin activity (Richardson et al., 1988).

Ricin has been crystallised and a high resolution 3-D structure has been determined (Montford et al., 1987). The A chain is a globular protein with extensive secondary struture. About 30% of the protein consists of α helices whilst there is only one region of extensive β sheet. The A chain folds into three domains. The amino

terminal 117 residues comprise the first domain, with the second and third domains encompassing residues 118-210 and 211-267 respectively. This C terminal domain contains the single cysteine (260) which is disulphide bonded to the B chain in ricin. In addition to this the A and B chains are held together by hydrophobic forces (Houston, 1980) whose association does not depend on the interchain disulphide bridge. The association of the two subunits has been shown to be entropically driven, a characteristic of hydrophobic interactions and it has been postulated that these are the main driving forces for the association with the disulphide bridge stabilising the subunits once they have associated (Lewis and Youle, 1986).

The B chain folds into two topolgically similar domains each containing a shallow galactose binding cleft, confirming earlier protein sequencing work which showed the B chain was the product of gene duplication (Villafranca and Robertus, 1981). Residues important to the binding of galactose in each of the clefts have been identified and site-directed mutagenesis has been used in this laboratory to delete some of these in order to try and abolish the sugar binding activity of the B chain.

The X-ray structure of ricin revealed a prominant cleft in the A chain which was thought likely to be the active site cleft. This was further borne out as more RIPs were sequenced and it became clear that some residues within this cleft were absolutely conserved in the various RIPs. Site-directed mutagenesis has begun to be used to try and define a role for each of these residues. However the analysis is complicated by the belief that the A chain undergoes a confirmational change when it is released from the B chain (Tasira

et al., 1978) and that these changes are responsible for the activation of the enzyme which is partially or totally inactive in ricin (Olsnes et al., 1976). Ricin A chain expressed in E.coli has been crystallised in order to investigate these changes but at present no structure has been determined (Robertus et al., 1987).

1.3.2 Function of the A and B Chains.

Early purifications of the ricin into its two constituent chains demonstrated that it was the A chain that was responsible for inhibition of protein synthesis in cell-free translation systems and that only the B chain could bind to erythrocytes (Olsnes and Pihl, 1973). Inhibition of protein synthesis could be demonstrated at very low ricin concentrations suggesting that it acted catalytically (Olsnes and Pihl, 1972a) and a number of authors subsequently showed that it was the 60S subunit that was the target for ricin A chain (Montanaro et al., 1973, Sperti et al.,1973). However the precise mode of action remained unknown; although no cofactors were required it was shown that the inhibition of protein synthesis was not associated with any RNase or protease activity (Mitchell et al., 1976).

The toxicity of ricin to whole cells was found to result from three sequential steps: (1) binding of the whole protein to the cell surface via the B chain; (2) penetration of the A chain into the cytosol, and (3) inhibition of protein synthesis. Ricin opportunistically binds to surface oligosaccharides terminating in galactose or N-acetylgalactosamine (Baeziger and Fiete, 1979) and

although eukaryotic cells have many potential binding sites on their surface (3 x 10^7 in Hela cells)(Sandvig et al., 1978), internalisation of the bound toxin is apparently very inefficient. So while it has been estimated that a single A chain is capable of killing a cell (Eiklid et al., 1980), several thousand molecules must bind to the cell surface to ensure the internalisation of this single molecule (Olsnes et al., 1979).

At present little is known about the mechanism by which ricin is internalised or the identity of the intracellular compartment from which the toxin enters the cytoplasm. It does however appear to be different from the mechanism by which diphtheria toxin enters the cell. In the latter case it has been shown that this is via an endocytotic vesicle with a low pH, probably an endosome (reviewed in Pappenheimer, 1977) and that the low pH induces a conformational change in the B chain of the diphtheria toxin. This exposes hydrophobic domains on the B chain which are capable of inserting into the membrane of the vesicle forming an ion-permeable channel through which the A chain is able to pass and enter the cytoplasm (Donovan et al., 1981, Kagan et al., 1981). In contrast, with ricin there is no evidence that low pH is necessary for transfer of the A chain into the cytoplasm and it is likely that ricin enters from a neutral but as yet unidentified vesicle (Sandvig and Olsnes, 1982). The mechanism by which the A chain is translocated out of this vesicle also differs from diphtheria toxin. There is no evidence that it is by way of an ion channel formed by the B chain although it does seem that the B chain plays a key role in facilitating A chain

translocation (Youle and Neville, 1982). As yet, how this is achieved or the domain on the B chain responsible remains unknown.

1.3.3 N-glycosidase Activity of Ricin A Chain.

In agreement with other results Endo and coworkers (1987) found that there was no ribonuclease activity associated with the ricin A chain catalysed inactivation of rat liver ribosomes. However they found that the 28S rRNA in the toxin-treated ribosomes had a slightly altered electrophoretic mobility. This altered mobility was also identified in an approximate 550 nucleotide fragment which was probably generated from the 28S rRNA by contaminating ribonucleases associated with the ribosomes during preparation. Direct RNA sequencing of the fragment showed that it represented the 3' terminal 553 nucleotides of the 28S rRNA and that ricin A chain modified both or either of G_{4323} and A_{4324}, resulting in these nucleotides becoming resistant to ribonuclease. Treatment of ricin modified rRNA with aniline at acidic pH resulted in the cleavage of the rRNA at a single position corresponding to the phosphodiester bonds surrounding A_{4324}. It was known that various amines, including aniline, and hydroxide ions cleave RNA strands by β-elimination if the base of the nucleoside residue is removed (Kochetov and Budovskii, 1972) and it was concluded that ricin A chain catalytically removed A_{4324} possibly by acting as an N-glycosidase. Thin-layer chromotography analysis confirmed that at catalytic amounts of ricin A chain, 1 mol of adenine was released per mol of ribosomes (Endo and Tsurugi, 1987). During the cleavage of this base no labelled phosphate was

incorporated into the rRNA and these authors concluded that ricin A chain inactivates ribosomes by cleaving the N-glycosidic bond of A_{4324} in 28S rRNA in a hydrolytic fashion (Endo and Tsurugi, 1987). The reaction is irreversible and apparently inhibited by free adenine, although no binding of adenine to ricin A chain was detected (Zamboni et al., 1989).

N-glycosidases had previously been reported and the substrates for these enzymes include NAD (Kaplan et al., 1951) and uridine (Magni et al., 1975). These proteins also catalyse irreversible reactions and are inhibited by the product of the reaction. However ricin A chain is unique even amongst these enzymes in that it catalyses the removal of a single base from over 7000 in eukaryotic rRNA.

The discovery of this activity in ricin A chain led to the subsequent investigation of the mode of action of other RIPs. A range of type 1 and type 2 plant RIPs were tested and shown to have exactly the same site of action in rat 28S rRNA as ricin A chain (Endo et al, 1988a, Endo et al., 1988b, Endo et al., 1988c, Endo et al., 1989). The related bacterial RIPs also act as N-glycosidases with the same specificity (Endo et al., 1988d) and it therefore seems likely that this activity is shared by all the members of this group of proteins.

The site of depurination in 28S rRNA is in a highly conserved sequence. It is conserved in plant 25S rRNA (Barker et al., 1988) and 26S rRNA (Kolosha and Fodor, 1990), yeast 26S rRNA (Veldman et al., 1981), Xenopus laevis 28S rRNA (Clark et al., 1984) and E.coli 23S rRNA (Brosius et al., 1980)(Fig. 1.3.1).

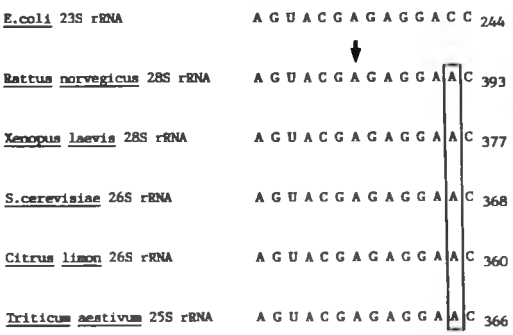


Fig. 1.3.1 Ricin A chain depurination site.

The base identified by Endo et al. (1987) as the site of depurination in rat 28S rRNA is arrowed. Differences in the sequence of the rRNAs compared to E.coli 23S rRNA are boxed. The numbers in subscript refer to the length in nucleotides from the 3' end of the rRNAs to the arrowed adenine. The sequences for these rRNAs are referenced in the text.

The adenine in this sequence which corresponds to the one identified by Endo in rat 28S rRNA has been found to be the site of action of ricin A chain in yeast 26S rRNA (Bradley et al., 1987, Stirpe et al., 1988) and Xenopus ribosomes (Saxena et al., 1989). This has led to the suggestion that all susceptible ribosomes (see section 1.7.1) are inactivated by cleavage of the same base in this sequence. There is however no direct evidence that the removal of the one base is sufficient to inhibit protein synthesis other than the fact that this is the only activity that has been identified with these proteins.

1.3.4 Sequence Conservation in Ribosome Inactivating Proteins.

The similarity in the enzymatic activity of both plant and bacterial RIPs is reflected in the high degree of homology at the amino acid level. The first comparison, between trichosanthin and ricin A chain identified 91 identical and 42 conservative residues (Xuejun and Jiahuai, 1986). Since then as more RIPs have been cloned and sequenced the list has been expanded to include abrin A chain, barley protein synthesis inhibitor and Mirabilis antiviral protein (Chow et al., 1990), as well as the Shiga-like toxins (Calderwood et al., 1987). The homology between these proteins is especially high in the residues which have been identified from the X-ray structure of ricin A chain as being clustered in the prominent cleft which is thought to be the active site of the enzyme. These correspond to Glu 177, Arg 180, Asn 209 and Trp 211 in the ricin A chain sequence. Selection of ricin A chain mutants which are no

longer toxic to Saccharomyces cerevisiae identified a high number of point mutations within these residues (Frankel et al., 1989) and also lends support to a role for these residues in the catalytic activity of ricin A chain. Interesting as well is the conservation of these residues in E.coli RNase H and Rous sarcoma virus pol gene polypeptides possibly reflecting the common feature of these proteins in RNA binding and the important role of the residues in this function (Ready et al., 1988).

Site-directed mutagenesis has begun to be used in a number of laboratories, including this one, to try and define a role for each of these residues. Hovde et al (1988) mutated Glu 167 of E.coli SLT-1 and found a 1,000-fold reduction in activity, whereas the same change of the equivalent residue Glu 177 in ricin A chain to Asp resulted in a 100-fold reduction in translation inhibiting activity (Schlossman et al., 1989). Mutations of Arg 180 to Lys or His had no and a 1,000-fold reduction in activity respectively. It is hoped that on the basis of these and future mutations it will be possible to propose a detailed mechanism for the activity of these proteins. However to date no such mechanism for ricin A chain or indeed any N-glycosidase has been worked out.

The importance of these residues in the catalytic activity of ricin A chain and the results of the mutations described above are in disagreement with previous work carried out in this laboratory (May et al., 1989). May et al. deleted a pentapeptide including Glu 177 and Arg 180 from ricin A chain and found that the protein was still active in a rabbit reticulocyte lysate. This work has however been repeated by J.Chaddock in this laboratory and the mutated ricin

A chain was found to be catalytically inactive. There does therefore seem to be a trivial explanation to resolve this difference of opinions on the importance of these residues.

1.4 THE EUKARYOTIC RIBOSOME.

1.4.1 Structure of the 80S Ribosome.

The substrate for ricin A chain catalysis, the eukaryotic cytoplasmic ribosome, is an approximate 4.3 MDa ribonucleoprotein complex with a sedimentation coefficient of approximately 80S. The two subunits, 40S and 60S consist of one molecule of 18S rRNA and approximately 30 proteins, and one molecule each of 5S, 5.8S and 25-28S rRNAs and approximately 45 proteins respectively (Wool, 1979). Data obtained by electron microscopy has shown that eukaryotic ribosomes have the same basic morphology as the prokaryotic 70S ribosome. However the detailed localisation of functional domains within the eukaryotic ribosome has been unsuccessful and to date this information is based largely on the assumed homology to the prokaryotic ribosome. The study of the eukaryotic ribosome has been hampered by the inability to reconstitute ribosomes *in vitro* and the general lack of well-defined genetics, both of which have been successfully employed to study the prokaryotic ribosome. The exception to this is the yeast ribosome where the ability to use genetic analysis meant that most of the early work on eukaryotic ribosomes was carried out on yeast. In particular the use of genetics with yeast identified a number of mutations in ribosomal proteins

which resulted in resistance to individual antibiotics (reviewed in McLaughlin, 1974). Yeast is however very much the exception when it comes to studies on the structure of the eukaryotic ribosome. Recently the use of techniques such as crosslinking with bifunctional reagents and ultraviolet irradiation, affinity labelling and immunoelectron microscopy have begun to correct this imbalence.

Early studies tended to concentrate on the position and role of the ribosomal proteins in the overall ribosomal function. This is understandable considering enzymes are proteins and so it was anticipated that the ribosomal proteins would carry out the enzymatic reactions of protein synthesis. Also the early studies on the resistance of E.coli to antibiotic inhibitors of protein synthesis identified mutations within the ribosomal proteins. The role of the rRNA was assumed to be purely structural acting to hold the important proteins in place. Over the past 5 years or so this situation has been all but reversed and the consensus of opinion now is that the rRNA has a fundamental role in all the enzymatic steps of protein synthesis.

The concept of catalytic rRNA is not a new one, having been proposed by Crick (1968) and Woese (1980) as an explanation for the problem of how the first translation apparatus evolved without the use of protein. Woese concluded that the basic mechanism of translation was at one time carried out by a collection of RNA molecules and it was only later in evolution that some of these functions were taken over by proteins. This transition in thinking was made easier by the discovery that RNA can have a catalytic activity. An RNA molecule (M1 RNA) has been shown to be essential for

the function of RNase P, the enzyme which processes transcripts of tRNA genes in prokaryotes (Gardiner and Pace, 1980) and the splicing of the rRNA precursor of Tetrahymena was shown to be an activity intrinsic to the RNA molecule (Bass and Cech, 1984). In both cases the RNA acts as an enzyme by binding to the substrate and promoting the rate and specificity of the reaction.

The role of rRNA in protein synthesis is supported by in vivo and in vitro studies which demonstrated that the removal of some individual proteins from E.coli ribosomes had no effect on the activity of the ribosomes (Dobbs, 1979, Garret, 1983). In addition the remarkable conservation of the structure of the rRNA throughout evolution and the findings that a number of inhibitors, including ricin A chain, act on the rRNA support the view that it has an important role in protein synthesis.

1.4.2 Structure of rRNA.

A large number of rRNAs from the ribosomes of eubacteria, archaebacteria and eukaryotes have been sequenced and comparisons made. Amongst each of the small subunit rRNAs (SSU-rRNA) and the large subunit rRNAs (LSU-rRNA) from all organisms there is a high degree of homology (reviewed in Noller, 1984 and Gutall and Fox, 1988). Of interest to the action of ricin A chain is the homology between the large rRNAs of eukaryotes and in particular the core regions which show the highest homology to the eubacterial 23S rRNAs. The differences between the eukaryotic LSU-rRNAs and the bacterial 23S rRNAs are predominantly found in the variable regions of

divergent domains which are scattered between the conserved core regions (Hassouna et al., 1984). Twelve divergent domains were identified in the mouse 28S rRNA. Within these divergent domains expansion or inserted sequences have been identified (Ware et al., 1983) and these account for the increase in length of the eukaryotic LSU-rRNAs ; 3392 nucleotides in yeast (Veldman et al., 1981), 4110 in amphibian (Ware et al., 1983) and 5025 in man (Gonzalez et al., 1985).

The 23S rRNAs consist of six major structural domains that are enclosed by long range base pairing (reviewed in Noller, 1984). The initial secondary structure for these domains proposed by Noller et al (1981) was based on the phylogenetic sequence comparisons and has subsequently been updated and altered (Noller, 1984, Leffers et al., 1987). Probing of E.coli 23S rRNA with ribonucleases (Garrett et al., 1984) and chemicals such as kethoxal and DMS (Leffers et al., 1988) has supported most of these secondary structure predictions and allowed the model to be refined. In particular the synthesis of individual domains in vitro using T7 RNA polymerase and cloned fragments of rDNA has allowed the structure of the free domains to be compared to the intact 23S rRNA; domain VI which includes the α-sarcin cleavage site is one such domain studied in this way (Leffers et al., 1988).

In the eukaryotic LSU-rRNAs similar long range base pairings are possible and domains can be folded in an identical manner in E.coli. By sequence homology to the E.coli model, secondary structures for yeast 26S rRNA (Veldman et al., 1981), rat 28S rRNA (Hadjiolov et al., 1984, Chan et al., 1983b), lemon 26S rRNA

(Kolosha and Fodor, 1990) and Xenopus laevis 28S rRNA (Clark et al., 1984) have been predicted. These eukaryotic LSU-rRNAs are folded into 7 domains with the 3' domain VII homologous to domain VI in E.coli 23S rRNA. Chemical probing of the conformation of yeast 26S rRNA has also shown the similarity in structure between this and E.coli 23S rRNA (Hogan et al., 1984).

A number of regions within the E.coli 23S rRNA have been assigned functions in protein synthesis on the basis of the results from studies of antibiotic resistance and site-directed mutagenesis of individual bases (Dahlberg, 1989). Moazed and Noller (1989) have published a detailed analysis of nucleotides which are located in the A, P and E sites (see section 1.5) of the 50S subunit. In agreement with earlier studies on single base mutations conferring resistance to specific antibiotic inhibitors of peptidyl transferase (Sigmund et al., 1984, Vester and Garrett, 1988) these bases are located almost exclusively in domain V and in particular in the central loop, which is thought to be the peptidyl transferase center of the ribosome (Steiner et al., 1988). The conservation in primary sequence and secondary structure in these regions suggests a similar function in eukaryotic protein synthesis.

1.4.3 Function of the Ricin A Chain Loop in Protein Synthesis.

The highly conserved sequence identified by Endo et al (1987) as the site of action of ricin A chain corresponds to positions 2653 to 2667 in E.coli 23S rRNA. These bases are located within a single stranded loop in domain VI (Wool, 1984) and a similar structure for

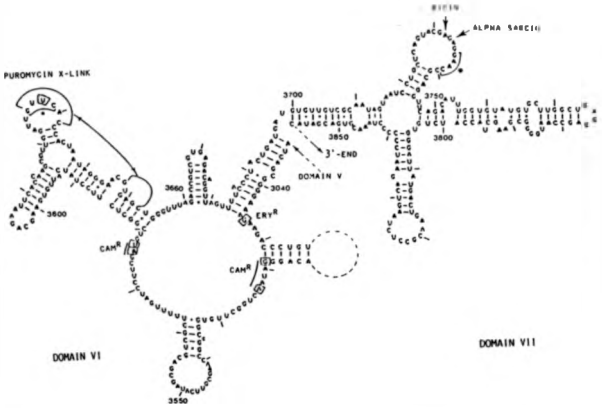


Fig. 1.4.1 The site of action of ricin A chain in the secondary structure of Xenopus laevis 28S rRNA.

The structure of part of the two domains shown is cited in Clark et al. (1984). The figure was reproduced from Endo (1988). The sequence GAAC, marked with a *, has been identified as a potential site of interaction between the rRNA and tRNA (Endo and Wool, 1982). Bases which are analogous to those identified in E.coli as important in resistance to certain antibiotics are also marked.

this sequence has been predicted in domain VII of eukaryotic LSU-rRNAs (Gutell and Fox, 1988)(Fig. 1.4.1).

The universal sequence homology within this loop led to the suggestion that it is of primary importance to the ribosomal function (Wool, 1984). This is supported by the action of the RIPs on this loop and the subsequent inhibition of protein synthesis that this causes. The functional importance of the structural integrity of this loop is in contrast to many other regions of rRNA. Ribosomes normally survive treatment where the rRNA is extensively nicked by non-specific nucleases (Kuechler et al., 1972, Cahn et al., 1970). Thus intact rRNA per se is not essential for protein synthesis. Indeed trypanosomes physiologically divide their 28S rRNA into domains by fragmentation into two large and four small molecules (White et al., 1986).

Moazed et al (1988) have provided direct evidence for the involvement of this loop in protein synthesis. These authors showed that when either of the two bacterial elongation factors, EF-G and EF-Tu were bound to 70S ribosomes a number of specific residues were protected from chemical modification by DMS and kethoxal, as monitored by primer extension. Both proteins protected G_{2655}, A_{2660} and G_{2661} whilst EF-Tu in addition protected A_{2665} and EF-G protected A_{1067} and A_{1069}. With the exception of the last two bases the other five lie within the conserved single-stranded loop; A_{2660} corresponds to the base depurinated by ricin A chain from eukaryotic LSU-rRNAs and G_{2661} is the site of cleavage of α-sarcin (Hausner et al., 1987). These authors concluded that this highly conserved sequence was the binding site for both factors, in agreement with earlier

competitive binding studies which showed that the two factors bound to the ribosome at overlapping sites (Richter, 1972). This conclusion is also substantiated by the results of the effect of ∝-sarcin on the subsequent binding of radioactively labelled EF-Tu and EF-G, which showed that the binding of both proteins was specifically blocked following cleavage after G_{2661} (Hausner et al., 1987). Site-directed mutagenesis has been used to try and confirm a role for these bases in the binding of these proteins. Tapprich and Dahlberg (1990) changed G_{2661} to cytosine in a high copy number plasmid containing the rrnB operon and although the results show the mutated ribosomes had an altered affinity for the EF-Tu-tRNA-GTP ternary complex, this was only in ribosomes with a second mutation in the ribosomal protein S12. Ribosomes with just the mutated base had no detectably altered function.

By analogy to the bacterial ribosome these results suggest that the ricin A chain-catalysed inhibition of protein synthesis on eukaryotic ribosomes is due to the alteration of the structure of a region of the rRNA which interacts with eEF-1 and eEF-2. The large number of studies carried out into the steps of protein synthesis inhibited in ricin A chain-treated ribosomes are also in broad agreement that it is the function of one or other or both of the elongation factors which is affected. However there is no consensus of opinion as to exactly which step of translation is inhibited and a number of conflicting results have been presented.

1.5 MECHANISMS OF PROTEIN SYNTHESIS.

1.5.1 General Considerations.

In the classical model of protein synthesis the ribosome contains two binding sites for tRNA called the A (aminoacyl or acceptor) and the P (pedtidyl or donor) site (Watson, 1964). The A site binds the incoming aminoacyl tRNA in the form of a tRNA-EF-Tu-GTP ternary complex whose anticodon must read the codon in the mRNA. A pedtide bond is formed resulting in the polypeptide being attached to the tRNA in the A site with the empty tRNA remaining in the P site. The cycle is completed by the translocation of the peptidyl-tRNA from the A site to the P site and the release of the deacylated tRNA. This last step is catalysed by the elongation factor EF-G and GTP.

The ribosome is highly flexible and its structure alters between a pre- and post-translocation conformation during protein synthesis. The two forms can be distinguished by their ability to bind the two elongation factors, but they are not dependant on the location of peptidyl-tRNA or mRNA (Nygard and Nilsson, 1989). The interconversion between these two forms has been suggested to be the driving force for translocation (Spirin, 1969). It has also been suggested that the inhibition of protein synthesis catalysed by toxins such as ricin A chain could be the result of locking the ribosome in one of these conformations (Nygard and Nilsson, 1990).

The two site model for protein synthesis has been challenged by evidence for the existence of a third site, the E or exit site, and a second tRNA entry site. The E site was characterised by its specific

affinity for deacylated tRNA (Rheinberger et al., 1981, Grajevskaja et al., 1982) and explains observations that there are often two tRNAs/ribosome in addition to the peptidyl-tRNA (Remne et al., 1989), an observation inconsistent with the classical two site model of protein synthesis. Furthermore it has been found that the occupation of the A site reduces the affinity for the E site and vice versa suggesting that the two sites interact allosterically (Gnirke et al., 1989). Transfer RNA bound to the E site interacts almost exclusively with the 23S rRNA (Moazed and Noller, 1989) in contrast to the A and P sites which are comprised of both the 50S and 30S subunits (Moazed and Noller, 1986, 1989).

Moazed and Noller (1989) have published evidence that the binding of the tRNA-EF-Tu-GTP ternary complex to the A site occurs in two steps, in agreement with earlier suggestions that there is a second entry site (Lake, 1977). Their conclusions were based on the observation that the protection of bases from chemical modification was different when tRNA was bound to the A site compared to when the dissociation of EF-Tu.GDP was prevented. Their model envisages that during the first step the anticodon loop of the tRNA interacts with mRNA and the 16S rRNA in the 30S subunit, while the EF-Tu moiety of the ternary complex interacts with the 23S rRNA. Following GTP hydrolysis EF-Tu.GDP is released and the binding of the tRNA to the A site is completed by the interaction of the aminoacyl end with the 23S rRNA in the peptidyl transferase center. This model neatly explains the inability of aminoacyl tRNA to participate in peptide bond formation when the release of EF-Tu is prevented (Wolf et al., 1977) and agrees with the kinetic proofreading models proposed by Hopfield (1974) to

account for the high accuracy of protein synthesis.

Eukaryotic protein synthesis follows the same overall mechanism as prokaryotic protein synthesis although some aspects are markedly different. The most marked differences are in the initiation step, the mechanism of elongation is essentially the same in both the 70S and 80S ribosomes, although the elongation factors are not interchangeable.

1.5.2 Initiation.

In contrast to the initiation of protein synthesis in prokaryotes, in eukaryotes more than ten different protein initiation factors (eIFs) are required compared to three in the former. Dissociation of the 80S into its two subunits is the first step and is catalysed by the binding of eIF-3 and eIF-4c to the 40S subunit (Trachel and Staehelin, 1979). This is followed by the binding of the initiator tRNA, met-tRNAi, GTP and eIF-2 to the 40S.eIF-3.eIF-4c complex to form a 43S preinitiation complex (Trachel and Staehelin, 1979). The next step is the binding of this complex to mRNA which involves another four eIFs. Unlike the situation in prokaryotes the position of the correct AUG codon is not identified by base pairing between the mRNA and the 16S rRNA (Hui and deBoer, 1987) but rather depends on the binding of the 43S preinitiation complex to the 5' cap structure on the mRNA and subsequent scanning of the mRNA (reviewed in Herman, 1989). Alternatives to this model have been found where the first AUG is not the correct initiating codon (Kozak, 1986) and here the correct AUG seems to be recognised by the direct binding of eIF-4b to the codon (Lutsch *et al.*, 1986). Once the initiation signal is identified

association of the 60S subunit to this complex takes place, catalysed by eIF-5 and the hydroylsis of GTP. Subsequent to this joining the factors associated with the preinitiation complex are released from the ribosome to leave the 80S initiation complex (Proud, 1986).

Little is known about the binding sites for the initiation factors and the requirements for ribosome association. Cross-linking studies have been carried out with eIF-2 (Westermann et al., 1980) and eIF-3 (Westermann and Nygard, 1983) and located the proteins at the interface of the 40S subunit in close proximately to each other. Furthermore some of the proteins cross-linked to eIF-2 have been identified as P site proteins (Uchiumi et al., 1986) suggesting that the met-tRNAi on the initiation complex is located at the future P site.

A number of inhibitors of initiation have been characterised; some act to stop the joining of mRNA to the preinitiation complex e.g. aurintricarboxylic acid (Huang and Grollman, 1972) whilst others inhibit the joining of the 60S subunit e.g. pactamycin (Kappen and Goldberg, 1976). Both of these inhibitors act on prokaryotic and eukaryotic ribosomes. In cell-free translation systems these inhibitors cause the breakdown of polysomes and the accumulation of free subunits.

1.5.3 Elongation.

Elongation in higher eukaryotes is catalysed by two factors eEF-1 and eEF-2 which are analogous to the prokaryotic factors EF-Tu and EF-G. eEF-1 activity is associated with a number of proteins. The low-molecular form is called eEF-1α, but this activity is also found in multiple higher molecular mass forms consisting of additional proteins,

designated eEF-1β, eEF-1γ and eEF-1δ (Carvalho et al., 1984). The precise function of these higher mass forms is unknown but the observation that they dissociate to leave eEF-1α upon germination in wheat germ has led to the suggestion that they may be storage forms (Sacchi et al., 1984). During both elongation factor-catalysed reactions GTP is hydrolysed and it has been suggested that they share a common GTPase center, involving the 5S rRNA.L5 complex (Grummt et al., 1974). The two factors bind to the ribosome at identical or partially overlapping binding sites, with eEF-1 binding to the post-translocation ribosomes and eEF-2 binding only to pre-translocation ribosomes carrying peptidyl-tRNA in the A site (Nolan et al., 1975, Nombela and Ochoa, 1973).

It has now been shown that eEF-2 is capable of forming two types of ribosomal complex; a high affinity pre-translocation complex is formed in the presence of nonhydrolysable GTP analogues whilst GDP promotes the formation of a low affinity post-translocation complex (Nilsson and Nygard, 1986, Nygard and Nilsson,1984). During translocation the hydrolysis of GTP is accompanied by a shift between these two forms, although the hydrolysis occurs after translocation in the low affinity complex, so the energy released is not directly involved in the translocation (Nilsson and Nygard, 1986). The GTPase activity of both eEF-1 and eEF-2 is intrinsic to both proteins but is activated by a common center on the ribosome (Nilsson and Nygard, 1984, Maessen et al., 1986).

Investigations into the partial reactions of protein synthesis on salt-washed yeast ribosomes identified a third factor that was absolutely required for polyphenylalanine synthesis (Skogerson and

Wakatama, 1976). This factor is only required for protein synthesis on yeast ribosomes. The other two yeast elongation factors are active with rat liver ribosomes in the absence of eEF-3 (Skogerson and Englehardt, 1977) suggesting that the activity of this third protein is provided by the ribosome itself in higher eucaryotes. To date no function has been attributed to this protein but the presence of this third elongation factor would suggest that the mechanism of elongation might be different in yeast.

A number of sequences from different organisms are known for eEF-1 (van Hemert et al., 1984, Nagata et al., 1984) and eEF-2 (Kohno et al., 1986, Oleinikov et al., 1989). They encode for approximately 50,000 and 95,000 Da proteins respectively. Both proteins have regions of homology which are shared by the two prokaryotic elongation factors and other GTP-binding proteins and which represent possible GTP binding and hydrolysis domains (Kohno et al., 1986). In addition amino acids 54-78 in eEF-2 and 57-81 in eEF-1 are homologous to sequences in the prokaryotic factors (van Hemert et al., 1984, Kohno et al., 1986) indicating that the region could have a common function in the elongation factors. Trypsin cleavage at Arg 66 within this sequence inhibits the binding of eEF-2 to pre-translocational ribosomes suggesting this region could be part of the contact area with the ribosome (Nilsson and Nygard, 1988). In agreement the accessibility of Arg 66 and a threonine which lies between 51 and 60 to trypsin attack and phosphorylation respectively were found to be different in the bound and unbound factor (Lavergne et al., 1990).

eEF-2 is ADP-ribosylated in the presence of diphtheria toxin and NAD at a unique modified histidine residue at position 715 and

results in the inactivation of the factor and inhibition of protein synthesis (van Ness et al., 1980). The modified factor is still able to bind to the ribosome but can not catalyse translocation (Davyodova and Ovchinnikov, 1990). This has been reported to be because the ribosylated factor is unable to form the high affinity complex with the ribosome but can still form the low affinity post-translocation complex (Nygard and Nilsson, 1985). The site of ADP-ribosylation is in the carboxyy-terminus of the protein and this has been taken as evidence that this region is also involved in the interaction of the enzyme with the ribosome (Kohno et al., 1986).

Cross-linking studies have identified a number of ribosomal proteins which may form the binding sites of eEF-1α (Uchiumi and Ogata, 1986) and eEF-2 on the ribosome (Uchiumi et al., 1986). Many of these proteins were also found to have an altered resistance to trypsin digestion after binding eEF-2 (Marzonki et al., 1990). However the direct interaction of these elongation factors with the rRNA has not been demonstrated in contrast to the case with E.coli 23S rRNA discussed in a previous section.

1.6 RICIN A CHAIN CATALYSED INHIBITION OF TRANSLATION.

1.6.1 Effects on Elongation and Initiation.

The initial studies into the effects of ricin A chain and related plant toxins on the partial reactions of protein synthesis in cell-free translation systems showed that they stabilised polysomes (Olsnes and Pihl, 1972a, 1972b). When inhibitors of initiation were

also included it was found that the runoff of already initiated peptides was inhibited by the toxins and the authors concluded that they acted by inhibiting a component of peptide chain elongation (Olsnes and Pihl, 1972b). However these studies in which most of the ribosomes were present in polysomes could not reveal whether the toxins had an additional effect on peptide initiation.

In contrast to the stabilisation of polysomes reported in cell free translation systems, intoxification of Hela cells (Grollman et al., 1974) and rats (Lin et al., 1972) with ricin resulted in polysome breakdown suggesting that initiation was inhibited. Subsequent to this Skorve et al. (1977) investigated the effect of abrin A chain on the initiation of protein synthesis in a wheat germ cell-free translation system. They showed that abrin A chain did not effect the binding of labelled methionine or labelled mRNA to the 40S subunit but that it caused a reduction in the amount of labelled mRNA in 80S monosomes and polysomes. In agreement with the 60S subunit being the target of these toxins this result suggested that abrin A chain inhibited the final step of initiation viz. the formation of the 80S initiation complex from the 40S preinitiation complex. To date this is the only published investigation of the effect of one of these toxins on protein synthesis inhibition in cell-free translation systems which shows that both initiation and elongation are inhibited. The majority of the work has concentrated on their effect on elongation and the particular step(s) of the elongation cycle which are inhibited. This is reflected by the fact that ricin is sold as an inhibitor of elongation in biochemical catalogues.

1.6.2 Inhibition of Elongation.

The ricin A chain inhibition of elongation has been the subject of a bewildering number of apparently contradictory reports and there is no consensus of opinion as to which of the steps are inhibited. A number of reviews have been published (Olsnes and Pihl, 1982, Vazquez, 1979) but there seems to be no one explanation for the differences in the results obtained by different groups.

There is general agreement that ricin A chain inhibits the binding of eEF-2 to ribosomes. This has been shown by measuring both the amount of labelled eEF-2 that can bind to ricin-treated ribosomes (Nolan et al., 1976, Brigotti et al., 1989, Montanaro et al., 1975) and also the amount of labelled nucleotides GTP, GDP and GMPPCP which bind in an eEF-2 dependant assay (Carrasco et al., 1975, Montanaro et al., 1975, Sperti et al., 1976). In addition the amount of ribosome-bound eEF-2 is diminished following treatment with ricin A chain (Nilsson and Nygard, 1986). This reduced amount of ribosome-bound eEF-2 reflects the inability of eEF-2 to form the high affinity pretranslocation complex; ricin A chain does not inhibit the binding of eEF-2 to the low affinity posttranslocation site on the ribosome (Nilsson and Nygard, 1986). This differential effect of the toxin on the two binding complexes of eEF-2 and the ribosome explains why some authors have reported a stimulation in the eEF-2-dependant GTPase on ricin A chain-treated ribosomes (Sperti et al., 1976, Nilsson and Nygard, 1986). This activity is associated with the low affinity complex (Nygard and Nilsson, 1989) so following treatment with ricin A chain when only this complex is able to be formed, the amount of GTP hydrolysis is increased.

Prior to treatment with ricin A chain the GTPase activity is lowered by the slow transition from the high affinity to the low affinity site. However this is at odds with the reports that the eEF-2 GTPase activity is inhibited by ricin A chain (Benson et al., 1975, Sperti et al., 1975), which could result from the inactivation of this ribosome center or indirectly as a result of the inability of eEF-2 to bind to the modified ribosome. At present there seems to be no explanation for these conflicting results on the effect of ricin A chain on the eEF-2 dependant GTPase activity.

It has been reported that ricin A chain, whilst inhibiting the binding of eEF-2 does not inhibit translocation. This is based on the reactivity of polysomes with labelled puromycin (Sperti et al., 1976, Fernandez-Puentes et al., 1976a) and also the reactivity of labelled phenylalanine bound to ribosomes with puromycin (Carrasco et al., 1975). In both cases prior incubation with ricin A chain had no effect indicating that both peptidyl transferase and one round of translocation were unaffected. Gessner and Irvin (1980) using the same assay of nonenzymatic binding of labelled Phe-tRNA to ribosomes showed that when ricin A chain was added prior to translocation phenylalanylpuromycin synthesis was inhibited whilst there was no inhibition if the toxin was added after translocation. This result confirms that the peptidyl transferase reaction of toxin-treated ribosomes is not inhibited, but contradicts the reports that translocation is unaffected. However this result does seem to be more in agreement with the accepted view that ricin A chain modified ribosomes are unable to bind eEF-2.

There are also differences in the reported affects of ricin A chain on the eEF-1 dependant binding of aminoacyl-tRNA to the ribosome.

It has been shown that ricin A chain strongly inhibits the poly(U)-directed binding of labelled Phe-tRNA to salt-washed ribosomes catalysed by eEF-1 (Carrasco et al., 1975, Olsnes et al., 1975b, Fernandez-Puentes et al., 1976a). Whilst other authors have reported no effect using the same assay (Sperti et al., 1976, Sperti and Montanaro, 1979, Brigotti et al., 1989). In agreement with these second results ricin A chain has been reported to have no effect on the binding of labelled eEF-1 to pretreated ribosomes (Nolan et al., 1976, Nilsson and Nygard, 1986).

Olsnes and Pihl (1982) have suggested that the differences may be due to the different levels of eEF-1 in the assays. At low eEF-1 the inhibition of Phe-tRNA binding was seen with ricin A chain but at high concentrations there was found to be no inhibition (Fernandez-Puentes et al., 1976a). They have suggested that where authors have been unable to show an effect on the binding of aminoacyl tRNA this was because the levels of eEF-1 were very high. On the otherside of the fence, Montanaro et al. (1978) and Sperti and Montanaro (1979) have argued that ricin A chain does not inhibit the eEF-1-dependant binding of aminoacyl-tRNA but that the inhibition observed by others was due to trace amounts of eEF-2 in the ribosome preparations. The presence of eEF-2 they argued would result in the formation of oligopeptides which in turn would lead to the overestimation of the number of control ribosomes carrying phenylalanyl tRNA. In the ricin A chain-treated ribosomes where the eEF-2 was inhibited these oligopeptides would not be formed and this would simulate the inhibition of Phe-tRNA binding. This elegant explanation is supported by the detection of these oligopeptides in the assays (Sperti and Montanaro, 1979) and the finding that if the ribosome preparations

were not treated with diphtheria toxin and NAD to inactivate trace amounts of eEF-2 an apparent inhibition of the eEF-1-dependant reactions is observed (Montanaro et al., 1978). However this disagreement is still not resolved; recent reports on the inhibition of protein synthesis by the related bacterial RIPs have found that the eEF-1 dependant binding of aminoacyl-tRNA is inhibited whilst there is no affect on the eEF-2 dependant translocation (Endo et al., 1988d, Igarashi et al., 1987).

1.6.3 Ricin A Chain Induced Conformational Changes.

The inhibition of protein synthesis catalysed by ricin A chain (Cawley et al., 1979, Skorve et al., 1977) and α-sarcin (Terao et al., 1988) can be partially overcome at high Mg^{++} concentrations. This effect is not due to the reduction in toxin activity at high Mg^{++}, which suggests that the high Mg^{++} concentrations are able to reverse toxin induced conformational changes in the ribosome whose structure is sensitive to the ionic conditions. Paleologue et al. (1986) have reported that ricin-treated 60S subunits have an altered thermal denaturation curve and taken together both these results suggest that the inactivation of the 60S subunit is associated with and possibly caused by conformational changes.

Terao et al. (1988) have studied the effect of both -sarcin and ricin A chain on the conformation of the ribosomes using a double labelling technique. They found that the labelling of certain proteins by radioactive N-ethylmaleimide was altered if the ribosomes were preincubated with either toxin. Ricin A chain specifically altered the

labelling of L14 whilst α-sarcin affected L3 and L4 suggesting that the toxins alter the conformation of ribosomes around these proteins. L3 and L4 have been located in the P and A site respectively and can be crosslinked to eEF-2 (Uchiumi et al., 1986) and so it is plausible that changes in the conformation of these important proteins could account for the observed inhibition of protein synthesis.

The observation that α-sarcin and ricin A chain alter the conformation at neighbouring but different sites is reflected in the different steps of protein synthesis which are inhibited. α-sarcin has been consistently reported to inhibit primarily the eEF-1-catalysed step of elongation whereas ricin A chain, as discussed in the previous section, appears to act on the eEF-2-catalysed step. This difference presumably reflects the greater disruption that occurs when the rRNA backbone is cleaved as opposed to the smaller effect of the removal of one base.

1.7 BINDING OF RICIN A CHAIN TO THE RIBOSOME.

1.7.1 Substrate Recognition.

Eukaryotic ribosomes from different organisms show a marked variation in their sensitivity to ricin A chain. Those from rat liver were found to be very sensitive whilst no inhibition of protein synthesis was found with wheat germ ribosomes at 5,000 times the concentration that was effective against the rat ribosomes (Cawley et al., 1977). The IC_{50}, the concentration giving a 50% inhibition of protein synthesis in a poly(U)-directed assay on salt-washed ribosomes,

for rat liver ribosomes has been published at 0.06 nM (Cawley et al., 1979). Protozoan ribosomes have also been reported to be relatively insensitive to inhibition by ricin A chain (Stirpe and Hughes, 1989, Cenini et al., 1988), whilst those from a variety of plants including Ricinus communis leaves are apparently resistant (Stirpe and Hughes, 1989). However this contradicts previous reports which found that plant ribosomes were sensitive to ricin A chain but only at high concentrations (Harley and Beevers, 1982). The contradictions apparent in the results from these experiments may be a reflection of the method used to measure the activity of ricin A chain on these ribosomes. In all cases this was done by measuring inhibition of protein synthesis on crude preparations of ribosomes in a poly(U)-directed assay for the incorporation of labelled phenylalanine. The level of protein synthesis in the control translations to which the toxin-treated translations were compared was often very low making meaningful comparisons difficult. In addition the crude preparations of plant 80S ribosomes would be expected to contain plastid ribosomes which are insensitive to ricin A chain (M.Hartley, unpublished) and which would presumably be active in the assays.

Ricin A chain is unusual in that it is inactive against E.coli ribosomes but reported to be active against some mitochondrial ribosomes which resemble the bacterial 70S ribosomes. So whilst ricin A chain was unable to inhibit protein synthesis dependant upon ribosomes from rat liver mitochondria (Greco et al., 1974) this process was inhibited in mitochondrial extracts from yeast (Lugnier et al., 1976). No other inhibitors, with the exception of the other RIPs have been found that are active against eukaryotic cytoplasmic and mitochondrial ribosomes

but not E.coli ribosomes.

The reason for the differential sensitivity of ribosomes to inactivation by ricin A chain and in particular the reason why E.coli ribosomes are insensitive is not immediately obvious since the nucleotide sequence and secondary structure around the ricin A chain site is highly conserved. Endo et al. (1987) showed that naked 28S rRNA, devoid of proteins, but retaining the secondary structure, could act as a substrate for ricin A chain-catalysed depurination and furthermore the Km for the reaction was the same as the intact ribosome, although the turnover number was considerably lower (Endo and Tsurugi, 1988). Thus ricin A chain appears to recognise a specific structure in rRNA and the recognition does not require ribosomal proteins. In agreement with this ricin A chain retains its specificity when tested on a synthetic oligoribonucleotide that mimics the stem loop around the site of depurination (Endo et al., 1988e). However the greatly reduced turnover rate of the reactions with naked 28S rRNA does suggest that whilst having no role in the binding of the toxin the ribosomal proteins play a part after the toxin has bound.

Surprisingly naked E.coli 23S rRNA in contrast to the intact ribosome is depurinated at the correct site by ricin A chain and the Km and Kcat for the reaction was almost the same as for naked 28S rRNA (Endo and Tsurugi, 1988). This is further evidence that ricin A chain recognises the secondary structure of the rRNA and also indicates that the insensitivity of E.coli ribosomes is because the loop is somehow protected from attack from ricin A chain by ribosomal proteins.

1.7.2 Protective Effect of Elongation Factors.

Several authors have shown that the inhibition of protein synthesis catalysed by ricin A chain can be overcome by the addition of saturating amounts of either eEF-2 (Olsnes et al., 1975b) or both eEF-2 and eEF-1 (Fernandez-Puentes et al., 1976a). It has been concluded that the damage caused by ricin A chain does not totally inactivate the ribosome but causes a decrease in the affinity for the factors i.e. at high concentrations of the factors the modified ribosomes can be forced to synthesise protein.

Preincubation of ribosomes with eEF-2 and GTP prior to the addition of ricin A chain has also been shown to reduce the subsequent inhibition of protein synthesis (Fernandez-Puentes et al., 1976b, Brigotti et al., 1989). Although this combination did not prevent the binding of labelled ricin A chain to ribosomes the preincubation of ribosomes with eEF-2 and the nonhydrolysable GTP analogue GDP(NH)P did reduce the binding of ricin A chain (Cawley et al., 1979). These authors suggested that eEF-2 and ricin A chain share the same or overlapping binding sites on the ribosome and that the binding of eEF-2 protects the ribosome from subsequent modification by ricin A chain. This protection could also account for the ability of high levels of eEF-2 and eEF-1 to overcome the inhibition of protein synthesis by ricin A chain, although it is not clear why on some occasions eEF-1 was found to be protective (Fernandez-Puentes et al., 1976a) but on others it had no affect (Fernandez-Puentes et al,. 1976b, Olsnes et al., 1975b).

Primary sequence comparisons between eEF-2 and ricin A chain identified a region of homology which when deleted from ricin A chain

resulted in the inactivation of the enzyme (May et al., 1989). Sequence homology might be expected between proteins which share a common binding activity and if this was the case then the sequence would be expected to be important for the full activity of the proteins. There is however no evidence to suggest that these conserved residues have a role in the binding of either protein to the ribosome. Indeed the region in eEF-2 which contains these six conserved amino acids is not in areas previously identified to be important in the binding of this protein to the ribosome.

The evidence of previous investigations suggests that the action of ricin A chain and eEF-2 are closely linked and possibly dependant on each other. So whilst ricin A chain modified ribosomes seem to be inhibited because they can no longer bind eEF-2, at the same time it seems that ricin A chain can only act if eEF-2 is not bound to the ribosome first.

1.8 AIMS OF THE PROJECT.

The preceeding sections of this chapter have outlined the present understanding of the mode of action of ricin A chain and the consequences of this to the function of the ribosome. At the onset of this project much of the detailed information on the N-glycosidase activity was not available and the site of action had only been shown in rat liver 28S rRNA. The elucidation of this mechanism by Endo and coworkers did however present the opportunity to investigate directly for the first time the activity of ricin A chain on ribosomes without having to measure the inhibition of protein synthesis. In particular work by Martin Hartley prior to my arrival had shown that the aniline assay, first described by Endo, could be used to determine the extent of depurination of rRNA by ricin A chain and that it would be a useful assay to investigate the action of ricin A chain on ribosomes. Consequently this assay forms the basis of much of the results presented in the following chapters.

The overall aims of the project were;

(1) to investigate the site of action of ricin A chain on eukaryotic ribosomes.

(2) to compare the differential sensitivity of ribosomes and the factors which affect the sensitivity of ribosomes to depurination.

(3) to investigate the partial reactions of protein synthesis inhibited by ricin A chain and in particular the effects of ricin A chain on initiation.

CHAPTER 2.

MATERIALS AND METHODS.

SECTION 2.1 MATERIALS.

2.1.1 Chemicals, Biochemicals, Radiochemicals and Enzymes.

The source of specific reagents is given below. All other chemicals were obtained either from BDH (AnalaR grade) or Fisons PLC depending on availabilty.

Amersham International PLC, Amersham, Buckinghamshire: [γ-^{32}P] dATP (> 3000 Ci/mmol), [α-^{32}P] dGTP (> 3000 Ci/mmol), [^{35}S] methionine (> 1300 Ci/mmol), [^{14}C] NAD (278 mCi/mmol), Amplify, T4 DNA ligase, DNA polymerase 1 (Kornberg), T4 polynucleotide kinase, all restriction endonucleases and placental RNAse inhibitor.

BDH Chemicals Ltd, Atherstone, Warwickshire: Amberlite monobed resin MB-3, bromophenol blue, aniline, 0.4 mm glass beads, ninhydrin and xylene cyanol.

Boehringer Corporation (London) Ltd, Lewes, East Sussex: calf intestinal phosphatase, calf liver tRNA, creatine kinase, creatine phosphate, deoxyribonucleotides, dideoxyribonucleotides, GMPPCP, puromycin and yeast tRNA.

Bio Rad Laboratories Ltd, Hemel Hempstead, Hertfordshire: hydroxylapatite.

Gibco-BRL, Paisley, Renfrewshire, Scotland: globin mRNA (rabbit), ultra-pure RNAse free sucrose.

Calbochem Corporation, La Jolla, California, USA: aquacide.

Difco Laboratories, Basingstoke, Hampshire: bacto-agar, bacto-peptone, bacto-tryptone and yeast extract.

Eastman Kodak, Rochester, New York, USA: N,N'-methylenebisacrylamide.

Fisons PLC, Loughborough, Leicester: acids and organic solvents.

ICN Biochemicals, High Wycombe, Buckinghamshire: diphtheria toxin.

Life Sciences Inc, St. Petersburg, Florida, USA: AMV reverse transcriptase.

Pall Biosupport, East Hills, New York, USA: Biodyne nylon membrane.

Pharmacia (UK) Ltd, Milton Keynes, Buckinghamshire: G-25 Sephadex, heparin-Sepharose and Micrococcal nuclease.

Schleicher and Schull, Dassel, West Germany: G1500 silica gel plates.

Sigma Chemical Co. Ltd, Poole, Dorset: agarose medium EEO (type II), ampicillin, adenine, aurintricarboxylic acid, anisomycin, bovine serum albumin (BSA), Coomassie brilliant blue, dithiothreitol

(DTT), DNAse 1 (bovine pancreas), ethidium bromide, E.coli tRNA synthetase, hexadecyltrimethylammonium bromide (CTAB), met-val dipeptide, L-amino acids, trypsin, trypsin inhibitor and uracil.

Whatman Labsystems Ltd, Maidstone Kent: DEAE-cellulose (DE 23), cellulose phosphate (P 11) and GF/C filters.

The recombinant ricin A chain used in these experiments was a kind gift from Dr R. Craig, ICI, Alderley Edge, Cheshire. It contains an N-terminal methionine prior to isoleucine, the N-terminal amino acid in planta. Apart from this the recombinant protein is identical to the native, plant purified A chain in primary sequence. The recombinant protein is non-glycosylated.

SECTION 2.2 GROWTH AND STORAGE OF BIOCHEMICAL MATERIALS.

2.2.1 Growth and Storage of Bacterial Stocks.

For short term storage the bacterial strains were maintained on L-agar plates (L-broth (1% (w/v) bacto-tryptone, 0.5% (w/v) yeast extract, 0.2M NaCl) solidified with 1.5% (w/v) bacto-agar). A loopful of overnight liquid culture was spread onto a plate and incubated at 37°C for 12-16 hours. Plates were then stored at 4°C for up to 2 weeks before restreaking. Bacterial liquid cultures were grown in L-broth at 37°C.

2.2.2 Growth and Storage of Yeast Stocks.

For the preparation of both yeast ribosomes and a cell-free translation system, the vacuolar protease-deficient strain ABYS 1 was used (Achstetter *et al.*, 1984). Storage and growth was carried out as described by Sherman *et al.* (1986). Cells were maintained at 4°C for up to 4 weeks on YPD plates (1% (w/v) yeast extract, 2% (w/v) bacto-peptone, 2% (w/v) glucose solidifed with 2% (w/v) agar) before restreaking. Yeast liquid cultures were grown at 30°C, 200 rpm in YPD supplemented with uracil and adenine (1% (v/v) each from 1mg/ml stocks).

2.2.3 Storage of Ribosome Inactivating Proteins.

Ribosome inacticating proteins (RIPs) were stored in sterile distilled water at 4°C.

SECTION 2.3 PREPARATION OF RIBOSOMES.

2.3.1 Yeast Ribosomes.

Yeast ribosomes were isolated from a yeast S-20 extract prepared by a modification of the method of Rothblatt and Meyer (1986). A 100 ml overnight culture of ABYS 1 was used to inoculate 2 x 500 ml of YPD, which was grown for 10-15 hr at 30°C, 200 rpm until the A_{600nm} was approximately 2. The cells were harvested in a Coolspin 6x500 rotor at 5000 rpm and 4°C for 10 min. The cell pellets were washed twice in sterile distilled water and resuspended in 3 volumes of lysis buffer (100 mM KOAc, 2 mM $Mg(OAc)_2$, 20% (v/v) glycerol, 20 mM Hepes/KOH, pH 7.4). PMSF (20 mg/ml in propan-2-ol) was added to a final concentration of 0.1 mg/ml and ½ volume of 40 mesh (0.4 mm) acid-washed glass beads were added, and the cells lysed by vigorous vortexing at 4°C. Cell lysis was checked using a microscope. The glass beads were pelleted by centrifugation at 2000 rpm and 4°C for 2 min in a Sorvall GSA swing-out rotor and the supernatant centrifuged for a further 20 min at 15,000 rpm and 4°C in a Beckman 8x50 rotor. The supernatant was carefuully removed and transfered into ultracentrifuge tubes, and the ribosomes pelleted at 50,000 rpm and 4°C for 3 hours in a Beckman 55.2 Ti rotor. The

pellets were washed twice in Endo buffer (25 mM Tris/HCl, pH 7.6, 25 mM KCl, 5 mM $MgCl_2$) and resuspended in a small volume of the same buffer. The ribosome concentration was estimated from the A_{260nm}, assuming that an A_{260nm} of 10= 1 mg/ml ribosomes (Wool, 1979). Typically the yield was approximately 10 mg ribosomes/100 ml culture.

2.3.2 Rabbit Reticulocyte Ribosomes.

Rabbit reticulocyte lysate (section 2.12.1) was layered over a 1 ml cushion of 1 M sucrose in Endo buffer, contained in a 3 ml centrifuge tube, and the ribosomes pelleted at 100,000 rpm and 2°C for 40 min in a Beckman TL-100 rotor. The pellet was rinsed twice in Endo buffer and resuspended in Endo buffer. 1 ml of lysate yielded approximately 750 µg of ribosomes.

2.3.3 Wheat Germ Ribosomes.

A crude extract from wheat germ, prepared according to the method of Anderson *et al.* (1983), was used as a source of ribosomes. To 20-30g of wheat germ (General Mills), an equal mass of acid-washed 40 mesh glass beads and 50 ml of grinding buffer (50 mM Hepes/KOH, pH 7.6, 100 mM KOAc, 1 mM $Mg(OAc)_2$, 2 mM $Ca(OAc)_2$, 6 mM DTT) were added. The wheat germ was ground in a mortar and pestle at 4°C, transfered to 50 ml Oakaridge tubes and centrifuged at 10,000 rpm and 4°C for 10 min in a Beckman 8x50 rotor. The supernatants were removed and recentrifuged at 18,000 rpm and 4°C for 10 min. The supernatants were pooled and transfered to ultracentrifuge tubes and

centrifuged at 55,000 rpm and 4°C for 2 hours in a Beckman 55.2 Ti rotor. The ribosome pellets were rinsed in wheat germ buffer (25 mM Hepes/KOH, pH 7.6, 60 mM KOAc, 2.5 mM $Mg(OAc)_2$) and resuspended in wheat germ buffer.

2.3.4 Salt-Washed Ribosomes.

To remove bound elongation factors and aminoacyl-tRNA ribosomes were washed in 0.5 M KCl (Blobel, 1971). Ribosomes prepared by the methods outlined in sections 2.3.1 - 2.3.3 were incubated for 1 hour on ice in 50 mM Tris/HCl, pH 7.5, 0.5 M KCl, 5 mM $MgCl_2$ at a concentration of 2 mg/ml. The ribosomes were then pelleted by centrifugation for 40 min at 100,000 rpm and 4°C in a Beckman TL-100.3 rotor, washed twice in the appropriate buffer and resuspended in either Endo buffer or wheat germ buffer.

2.3.5 Storage of Ribosomes.

Purified ribosomes were stored in either Endo buffer or wheat germ buffer at -70°C at a concetration of 10-20 $\mu g/\mu l$.

SECTION 2.4 NUCLEIC ACID ISOLATION.

2.4.1 Isolation of RNA from Ribosomes and Cell-Free Translation Systems.

RNA was extracted by a combination of phenol and detergent. Typically reaction mixtures were diluted to 100 µl with H_2O and 10 µl 10% (w/v) SDS was added. Total RNA was extracted with an equal volume of TE-equilibrated (10 mM Tris/HCl, pH 8.0, 1 mM EDTA) phenol:chloroform (1:1 (v/v)) and ethanol precipipated by the addition of 1/10 th volume 2 M NaOAc/acetic acid, pH 6.0 and 2½ volumes ethanol. RNA was left to precipitate overnight at -20°C and pelleted by centrifugation at 11,000 rpm and 4°C in a microcentrifuge. The RNA pellet was washed in 70% (v/v) ethanol, dried *in vacuo* and dissolved in 10 ul of sterile distilled water. The rRNA concentration was estimated from the A_{260nm}, assuming that a 1 mg/ml solution of RNA has a A_{260nm} of 25 (Maniatis *et al.*, 1982). RNA was stored at -70°C.

2.4.2 Large Scale Plasmid DNA Isolation.

The method used was as described by Birnboim and Doly (1979). A 10 ml culture of L-broth, supplemented with 50 µg/ml ampicillin, was inoculated with a single *E.coli* transformant and incubated overnight at 37°C with shaking at 200 rpm. This was then used to inoculate a further 600 ml of L-broth supplemented with 50 µg/ml ampicillin, which was grown overnight at 37°C with shaking at 200

rpm. Cells were pelleted by centrifugation at 7000g and 4°C for 5 min in MSE 300 ml polypropylene tubes and resuspended in 3 ml of GET (25 mM Tris/HCl, pH 8.0, 50 mM glucose, 10 mM EDTA) per 600 ml culture. Cells were lysed by the addition of 200 ul GET containing 40 mg/ml lysozyme followed by a 5 min incubation on ice. Two volumes of 0.2 M NaOH, 1% (w/v) SDS was added and the tubes vortexed and left on ice for 5 min. ½ volume of 3 M NaOAc/acetic acid, pH 4.8 was added and after 30 min on ice, the tubes were centrifuged at 11,000g and 4°C for 15 min. The supernatants were removed and 0.54 volumes propan-2-ol added to precipitate nucleic acids. After 10 min at room temperature the precipitate was collected by centrifugation at 11,000g and 4°C for 10 min and the pellet redissolved in 3.2 ml of 2 M NH_4OAc. The samples were then recentrifuged as before, the supernatants were removed and 2.1 ml propan-2-ol was added to each. The tubes were incubated at room temperature for 10 min and the nucleic acids pelleted by centrifugation as above. The plasmid pellets were washed with 70% (v/v) ethanol, dried *in vacuo* and dissolved in 20 ml TE.

Plasmid DNA was further purifed by CsCl density gradient centrifugation (Maniatis *et al*, 1982). 23.76g CsCl was dissolved in each 20 ml plasmid solution, 4 ml of 5 mg/ml ethidium bromide was added and the mixtures loaded into 37 ml Beckman quickseal tubes. The tubes were filled with liquid parafin, balanced and sealed. Gradients were formed by centrifugation in a Beckman Vti 50 rotor at 45,000 rpm and 20°C for 16 hours. Plasmid bands were visualised under long-wave ultraviolet light and removed with an 18 guage needle. Ethidium bromide was removed by repeated extractions with propan-2-ol

saturated with CsCl-saturated TE. 4 volumes TE were added and the DNA ethanol precipitated with 1/10 th volume 3 M NaOAc/acetic acid, pH 4.8 and 2½ volumes ethanol. The pellet was washed in 70% (v/v) ethanol, dried in vacuo and dissolved in 500 µl TE.The concentration was estimated from the flouresence following electrophoresis on a 1% (w/v) agarose/1x TAE gel when compared to known amounts of the plasmid.

2.4.3 Small Scale Plasmid DNA Isolation.

This method was used to prepare plasmid DNA for preliminary restriction endonuclease analysis and was also a modification of alkaline lysis approach of Birnboim and Doly (1979). 10 ml cultures were inoculated with a single colony and grown overnight as described in section 2.2.1. 1.5 ml aliquots were removed from each culture and the cells pelleted in a microcentrifuge for 1 min. Each pellet was resuspended in 80 µl GET and 5 µl of 40 mg/ml lysozyme was added, followed by a 10 min incubation on ice. 160 µl 0.2 M NaOH, 1% (w/v) SDS was added and following a further 10 min on ice, 100 µl 3 M NaOAc/acetic acid, pH 4.8 was added. Chromosomal DNA was pelleted in a microcentrifuge and the supernatants removed. To each an equal volume of TE-equilibrated phenol:chloroform (1:1 (v/v)) was added and the DNA extracted and ethanol precipitated by the addition of 1/10th volume 3 M NaOAc/acetic acid, pH 4.8 and 2½ volumes ehtanol. The DNA was pelleted by centrifugation for 10 min in a microcentrifuge, dried in vacuo and dissolved in 50 µl TE.

2.4.4 M13 Phage Replicative Form DNA Extraction.

A 10 ml culture of L-broth inoculated with E.coli TG1 cells was grown overnight to act as 'feeder' cells. To a second 5 ml culture, a few drops of the overnight 'feeder' cells were added and the culture infected with a single phage colony. This was grown overnight at 37°C, 200 rpm and the cells harvested by centrifugation (see section 2.4.2). The cells were resuspended in 180 µl SET (100 mM Tris/HCl, pH 8.0, 50 mM EDTA, 20% (w/v) sucrose) and 180 µl of 4 mg/ml lysozyme in SET was added. The tubes were left at room temperature for 5 min , 300 µl 10% (w/v) Triton X-100 added and heated to 100°C for 2 min, followed by 1 min on ice. Cell debris was pelleted by centrifugation in a microcentrifuge for 10 min and 300 µl 7.5 M NH_4OAc was added to each supernatant. The tubes were incubated on ice for 30 min and the precipitated chromosomal DNA removed by centrifugation. 630 ul propan-2-ol was added to each supernatant and following 10 min at -20°C, the nucleic acid was pelleted in a microcentrifuge for 10 min. The pellets were washed with 70% (v/v) ethanol, dried in vacuo and dissolved in 60 µl TE.

SECTION 2.5 NUCLEIC ACID MODIFICATION AND RESTRICTION REACTIONS.

2.5.1 Treatment of RNA with Aniline.

The cleavage of ricin A chain-depurinated rRNA was first described by Endo et al. (1987). The method used here was a modification of this (May et al., 1989). 2-4 µg RNA in 1-2 µl was

incubated at 60°C for 2 min with 20 µl 1 M aniline/acetic acid, pH 4.5. 2 µl 7 M NH_4OAc and 65 µl ethanol were added and RNA precipitated on dry ice in the dark for 30 min. RNA was pelleted by centrifugation in a microcentrifuge for 15 min at 4°C, washed in 70% (v/v) ethanol and dried in vacuo. The pellets were then dissolved in 20 ul of 60% formamide in 0.1 x E buffer (3.6 mM Tris, 3 mM NaH_2PO_4, 0.2 mM EDTA).

2.5.2 Restriction Endonuclease Digests.

Digestion of plasmid and M13 replicative form DNA was carried out using the 10x buffers supplied by Amersham Internationl with it's enzymes. To check plasmid preparations, approximately 0.2 µg was cut in a final volume of 10 µl with 10 U of enzyme for 1 hour at 37°C. For the gel isolation of the EcoR1/Bcl 1 fragment from pGem26I 10 µg was cut with 20 U of each enzyme in a final volume of 50 µl for 2 hours at 37°C.

2.5.3 Dephosphorylation of Plasmid Vector.

For the subcloning of the EcoR1 fragment E of S.carlsbergensis 26S rRNA gene (Veldman et al., 1981) the vector pGem-3Z was used (see appendix 1). 1 µg of plasmid was linearised with EcoR1 (section 2.5.2) and the digest extracted with an equal volume of TE-equilibrated phenol:chloroform (1:1 (v/v)). The DNA was precipitated by the addition of 1/10th volume 3 M NaOAc/acetic acid, pH 4.8 and 2½ volumes ethanol. This was incubated on dry ice for 30 min and the DNA

pelleted by centrifugation in a microcentrifuge for 10 min. The DNA was dried *in vacuo* and redissolved in 20 ul of sterile distilled water. The 5' terminal phosphate groups were removed from the linearised vector using the method described by Maniatis *et al.* (1982). To the DNA was added 2.5 µl of 10x phosphatase salts (10 mM $MgCl_2$, 1 mM $ZnCl_2$), 2.5 ul 0.5 M glycine (pH 9.4 with NaOH) and 1 µl of 1 U/µl calf intestinal phosphatase. This was incubated for 30 min at 37°C and then extracted twice with an equal volume of TE-equilibrated phenol:chloroform (1:1 (v/v)). The DNA was precipitated, centrifuged and the pellet dried *in vacuo* as above. The pellet was dissolved in 100 µl of TE and 1 µl run on an agarose gel (section 2.6.2) to check for complete linearisation and estimate the concentration of DNA.

2.5.4 Ligation of DNA.

The EcoR1 fragment E of *S.carlsbergensis* excised from replicative form M13 was ligated to dephosphorylated pGem-3Z in 10 µl of 66 mM Tris/HCl, pH 7.5, 5 mM $MgCl_2$, 10 mM DTT and 1 mM ATP. 1 µl of 2.5 U/µl T4 DNA ligase was added and the reaction mix incubated at room temperature for 4 hours. In each reaction there was approximately 10 ng each of the vector and insert.

SECTION 2.6 ELECTROPHORESIS OF NUCLEIC ACIDS.

2.6.1 Formamide-agarose Gels.

Ribosomal RNA was fractionated on 1.2% (w/v) agarose, 0.1x E buffer (3.6 mM Tris,3 mM NaH_2PO_4,0.2 mM EDTA), 50% formamide gels run in Leicester Biocenter gel tanks. Samples of rRNA treated with aniline (section 2.5.1) were heated to 65°C for 5 min to denature the RNA and then cooled on ice. 3 µl of 50% (v/v) glycerol, 0.1% (w/v) bromophenol blue was added to each sample and the samples loaded onto the gels (100 ml in 15 x 15 cm plates). The gels were electrophoresed at 20 mA constant current for 2-3 hours in 0.1x E buffer. To visualise the RNA, gels were soaked for 10-20 min in 2 ug/ml ethidium bromide, destained in distilled water for 30 min and viewed on a UV transilluminator. Gels were photographed with a Polaroid instant camera using either Polaroid 665 or 667 film.

2.6.2 Agarose Gels.

For the analysis of restriction endonuclease digests and the isolation of DNA fragments onto dialysis membrane (section 2.7.1), 1% (w/v) agarose gels in 1x TAE (40 mM Tris/acetate pH 8.0, 1 mM EDTA) were used (Maniatis et al., 1982). The appropriate weight of agarose was melted in 1x TAE, allowed to cool to 60°C and ethidium bromide added to 0.1 µg/ml. Gels were run in BRL mini-gel tanks. Before loading 1/5 th volume 5x loading buffer (50% (v/v) glycerol, 50 mM EDTA, 0.1% (w/v) bromophenol blue) was added to each sample.

Gels were run submerged in 1x TAE for 30-60 min at 70 mA constant current and visualised and photographed as described in section 2.6.1.

2.6.3 Polyacrylamide Sequencing Gels.

The products of primer extension reactions were run on 8% (w/v) polyacrylamide,7.5 M urea gels (Maniatis et al.,, 1982). A stock solution of 38% (w/v) acrylamide, 2% (w/v) N,N'-methylenebisacrylamide, deionised with Amberlite monobed resin MB1, was used to prepare the working solution for the gels. For a 40 cm gel, 21g of urea was dissolved in 10 ml 40% (w/v) acrylamide (1:19 bis), 5 ml 10x TBE (89 mM Tris base, 89 mM boric acid, 2 mM EDTA) made up to 50 ml with sterile distilled water. The acrylamide was polymerised by the addition of 70 µl freshly prepared 10% (w/v) ammonium persulphate and 70 µl TEMED, and immediately poured. Samples were heated to 95°C for 3 min prior to loading and electrophoresis was carried out at 40W in 1x TBE. Following electrophoresis gels were fixed in 10% (v/v) methanol, 10% (v/v) acetic acid for 15 min, transfered to Whatman 3MM paper and dried in vacuo at 80°C for 1 hour. Autoradiography was carried out overnight at room temperature using Fuji X-ray film.

SECTION 2.7 ISOLATION OF DNA FOLLOWING ELECTROPHORESIS.

2.7.1 Elution onto Dialysis Membrane.

The 422 bp EcoRI/BclI fragment cut from pGem26I was isolated from a 1% (w/v) agarose, 1x TAE gel by elution onto dialysis membrane (Maniatis et al., 1982). The band was visualised using a long-wave length UV light and an incision made directly ahead of the band. A piece of Whatman 3MM paper backed by a piece of dialysis membrane was placed in the cut, with the paper facing the DNA band and electrophoresis continued until the band had migrated onto the paper. The paper and the dialysis tubing were placed in a 400 µl Eppendorf tube with a hole pierced in the bottom, which in turn was placed in a 1.5 ml Eppendorf tube. This set up was centrifuged for 15s in a microcentrifuge and the eluate from the 1.5 ml Eppendorf recovered. 100 ul of elution buffer (50 mM Tris/HCl, pH 7.6, 0.2 M NaCl, 1 mM EDTA and 0.1% (w/v) SDS) was added to the smaller Eppendorf tube and both tubes recentrifuged for 15s. The eluate was recovered and pooled with the first eluate. This procedure was repeated and the total eluate extracted twice with TE-equilibrated phenol:chloroform (1:1 (v/v)). The DNA was ethanol precipitated (section 2.5.3), washed in 70% (v/v) ethanol, dried in vacuo and dissolved in 10 ul TE. The DNA concentration of the fragment was estimated from it's flouresence following electrophoresis on a 1% (w/v) agarose/ 1x TAE gel using known amounts of λ Hind III markers as standards and comparing to the nearest sized band

SECTION 2.8 BACTERIAL TRANSFORMATIONS.

2.8.1 Competent Cells.

Competent TG1 and GM119 cells were made by a method based on that of Mandel and Higa (1970). A single colony of bacteria was used to inoculate a 10 ml culture of L-broth which was grown overnight at 37°C, 200 rpm. This was then used to seed a further 50 ml of L-broth which was grown under the same conditions until the A_{550nm} was approximately 0.48-0.5. The cells were harvested at 5000 rpm and 4°C in a Beckman 8x50 rotor, resuspended gently in 0.5 volumes of ice cold 50 mM $CaCl_2$ and incubated on ice for 30 min. The cells were repelleted as before and resuspended in 2 ml ice-cold 50 mM $CaCl_2$. Competent cells were kept on ice for 1 hour before use or frozen in liquid nitrogen and kept at -70°C in 200 ul aliquots.

2.8.2 Transformation of E.coli with Plasmid DNA.

Normally two transformations were carried out for each ligation reaction; one with 5 µl of the reaction mix and the other with 1 µl. A third mock transformation was carried out without DNA. 200 µl of competent cells were mixed with the appropriate amount of DNA and left on ice for 40 min. The cells were heat-shocked at 42°C for 2 min and 1 ml of L-broth was added to each. The cells were incubated at 37°C for 1 hour, centrifuged in a microcentrifuge for 2 min and resuspended in 100 µl L-broth. The cell suspensions were then plated

out onto 1.5% (w/v) L-agar plates containing 50 µg/ml ampicillin and incubated overnight at 37°C.

2.8.3 Transformation of E.coli with Single Stranded M13 DNA.

To prepare replicative form M13 DNA competent TG1 cells were transformed with single stranded M13 phage. The method used was similar to the one for plasmid transformation, except that following the heat-shock cells were added to 3 ml B-Top (1% (w/v) bacto-tryptone, 0.5% (w/v) NaCl, 0.8% (w/v) agar) kept at 42°C. 100 µl of an exponentially growing culture of TG1 was added to act as 'feeder' cells and the mixture poured onto dry L-agar plates. When the B-Top had set the plates were inverted and incubated overnight at 37°C.

SECTION 2.9 RADIOLABELLING OF DNA AND RNA IN VITRO.

2.9.1 Nick Translation of DNA Fragments.

To prepare a radioactive probe for Northern blots the 422 bp EcoR1/Bcl 1 fragment from pGem26I was labeled by nick translation (Maniatis et al., 1982). 5 ug of the gel isolated fragment (section 2.7.1) was chilled on ice in a final volume of 10 µl containing 1 nmol each of dATP, dCTP, dGTP and dTTP, 20 uCi [α-^{32}P] dGTP, 50 mM Tris/HCl, pH 7.2, 10 mM $MgSO_4$, 0.1 mM DTT and 50 µg/ml BSA. 0.5 µl diluted DNAse 1 holoenzyme (0.1 µg/ml) was added and the reaction mixture gently mixed. 1 µl of E.coli DNA polymerase 1 (5 U/µl) was added and the reaction incubated for 1 hour at 15°C. The

DNA was extracted once with TE-equilibrated phenol:chloroform (1:1 (v/v)) and ethanol precipitated. The pellet was dried *in vacuo* and redissolved in 100 µl of sterile distilled water. The specific acticity of the probe was estimated at 4×10^7 dpm/µg.

2.9.2 Labelling 5' Ends of DNA with T4 Polynucleotide Kinase.

To label the 17 nucleotide oligonucleotides used as primers for reverse transcriptase a simplified version of the method described by Maniatis *et al.* (1982) was used. Since the oligonucleotides were supplied with no 5' phosphate groups, dephosphorylation of the DNA was not necessary. The reaction mixtures contained the following in a final volume of 10 µl; 100 ng oligonucleotide, 50 mM Tris/HCl, pH 8.0, 10 mM $MgCl_2$, 5 mM DTT and 10 µCi [γ-^{32}P] dATP. To this 0.5 µl T4 polynucleotide kinase was added and the reaction mixtures incubated at 37°C for 30 min. To stop the reaction 1 µl 7 M NH_4OAc and 27 µl ethanol were added and the tubes placed at -20°C for 30 min. The oligonucleotides were pelleted in a microcentrifuge for 15 min at 4°C, dried *in vacuo* and dissolved in 10 µl sterile distilled water.

2.9.3 Labelling Met-tRNAi.

E.coli tRNA synthetase is capable of charging only the initiator $tRNA^{met}$ ($tRNAi^{met}$) from a mixture of eukaryotic tRNA's (Ghosh *et al.*, 1971) and this can be exploited to specifically label eukaryotic $tRNAi^{met}$ with ^{35}S methionine. The conditions used were as

described by Clemens et al. (1984) except that commercially available purified E.coli tRNA synthetase was used, instead of a crude E.coli extract. The reaction mixtures contained the following in a final volume of 1 ml; 50 mM Hepes/KOH, pH 7.5, 50 mM KCl, 8 mM $Mg(OAc)_2$, 2 mM DTT, 4 mM ATP, 1 mM CTP, 100 uCi [^{35}S] methionine, 1 mg calf liver tRNA and 500 U E.coli tRNA synthetase. The reaction mixtures were incubated at 30°C for 30 min and the tRNA extracted with an equal volume of TE-equilibrated phenol. The tRNA was ethanol precipitated, pelleted and dried in vacuo (as for section 2.9.2). The pellets were dissolved in 300 µl 20 mM NaOAc/acetic acid, pH 5.0 and stored in small aliquots at -70°C. The specific activity was estimated from directly counting an aliquot at 90,000 dpm/µg.

SECTION 2.10 NORTHERN BLOTTING AND HYBRIDISATION.

2.10.1 Transfer of RNA onto Nylon Membrane.

RNA was fractionated in a 1.2% (w/v) agarose, 50% formamide, 0.1x E buffer gel (section 2.6.1) and transfered to a Biodyne nylon membrane by capillary blotting (Maniatis et al., 1982). Gels were soaked for 1 hour in 20X SSC (3 M NaCl, 0.3 M trisodium citrate, pH 7.0) and transfer achieved overnight in the same buffer. Following transfer, membranes were air-dried and baked in vacuo at 80°C for 1 hour to fix the DNA.

2.10.2 Hybridisation of the Nick Translated Probe.

Filters were prehybridisation for 6 hours at 42°C in 5X SSPE (0.75 M NaCl, 75 mM Na phosphate, pH 7.0, 5 mM EDTA), 5X Denhardts (0.1% (w/v) ficoll, 0.1% (w/v) polyvinylpyrolidone, 0.1% (w/v) BSA), 100 ug/ml denatured/sheared salmon sperm DNA, 0.3% (w/v) SDS and 50% (v/v) formamide. Hybridisation was carried out for 12-16 hours under the same conditions with 0.75 µg (3 x 10^7 dpm) of the nick translated probe (section 2.9.1). Filters were washed for 30 min in 2X SSC, 0.1% (w/v) SDS at room temperature, followed by 30 min in 0.1X SSC, 0.1% (w/v) SDS at 50°C. The filters were air-dried and autoradiographed at -70°C for 2-3 days.

SECTION 2.11 PRIMER EXTENSION.

2.11.1 Annealing of Primer and Extension.

Annealing of the primers and extension was carried out by a modification of the method of Moazed et al. (1986) as described in May et al. (1989). 10 ng of end-labelled oligonucleotide was annealed to 4 µg of total RNA in 7.5 µl of 50 mM Hepes/KOH, pH 7.0, 5 mM Na boroate and 0.1 M KCl. 1 µl of this reaction mixture was subsequently used in each of the extension reactions. For the dideoxysequencing reactions the appropriate dideoxynucleotide was included at a final concentration of 25 µM. The reactions were stopped by the addition of 1 µl loading dye (0.25% (w/v) bromophenol blue, 0.25% (w/v) xylene cyanol, 30% (v/v) glycerol).

Electrophoresis and autoradiography were carried out as described in section 2.6.3.

SECTION 2.12 CELL-FREE TRANSLATION SYSTEMS.

2.12.1 Rabbit Reticulocyte Lysate.

The lysate used was a generous gift from Dr T. Hunt and was supplied already supplemented with 20 μM haemin and 50 μg/ml creatine kinase. Where indicated in the figure legends the lysate was treated with Micrococcal nuclease to remove endogenous mRNA (Jackson and Hunt, 1983). Reactions were usually carried out in 50 μl total volume containing 40 μl lysate, 2.5 μl 'KM' salts (2 M KCl, 10 mM $MgCl_2$), 2.5 μl 0.2 M creatine phosphate and 2.5 μl amino acid mix (2.5 mM each of the 19 amino acids minus methionine). Reactions were preincubated for 5-10 min at 30°C with or without the addition of inhibitor, 2.5 μl globin mRNA (50 μg/ml) and 2 μl [^{35}S] methionine (15 μCi/μl) or 2 μl [^{35}S] met-tRNAi (approximately 180,000 dpm) were added and the incubations continued for the times indicated in the figure legends. The incorporation of [^{35}S] methionine into TCA insoluble protein was determined using the method of Jackson and Hunt (1983). Whatman GF/C filters were counted in 4 ml LKB Optiphase Safe scintillation fluid in an LKB 1212 Minibeta counter.

2.12.2 Yeast Lysate.

A yeast lysate was prepared from the vacuolar protease deficient strain ABYS 1 using the modified method of Rothblatt and Meyer (1986). The same protocol described earlier for the purification of yeast ribosomes (2.3.1) was used to make a crude S-20 lysate. 6x500 ml cultures were grown up to an A_{600nm} of 1.8 and harvested as before. The cells were fractured using a Braun glass bead homogeniser rather than vortexing by hand as this was found to be more efficient for the larger number of cells. Ultracentrifugation was carried out at 40,000 rpm and 4°C in a Beckman SW 50 rotor for 30 min. Although this results in large polysomes being pelleted it is necessary to remove an inhibitor of protein synthesis which also pellets with this fraction (Rothblatt, personnal communication). The supernatants were loaded onto a 1.5 cm X 10 cm Sephadex G-25 column equilibrated in lysis buffer and the column developed in the same buffer. Fractions with an A_{280nm} of greater than 0.8 were pooled and stored in 100 µl aliquots at -70°C.

The basic reaction mixture contained 20 µl S-100 lysate, 42 mM Hepes/KOH, pH 7.4, 190 mM KOAc, 2.8 mM $Mg(OAc)_2$, 20 mM creatine phosphate, 4 µg creatine kinase, 1 mM ATP, 0.1 mM GTP, 30 µM each of the 19 amino acids (minus methionine), 25 µCi [^{35}S] methionine and 50 U placental ribonuclease inhibitor in a final volume of 50 µl. Reactions were incubated at 25°C.

Incorporation of [^{35}S] methionine into TCA insoluble protein was estimated from 1 µl aliquots spotted onto Whatman No 1 discs. The discs were washed twice in 10% (w/v) ice-cold TCA and then for 15 min

at 90°C in 5% (w/v) TCA. The washed discs were rinsed in ethanol, air-dried and counted in 4 ml of LKB Optiphase Safe scintillation fluid.

SECTION 2.13 ANALYSIS OF CELL FREE TRANSLATION PRODUCTS.

2.13.1 Analysis of Proteins by PAGE.

Proteins were analysed on 10% or 15% SDS polyacrylamide gels as described by Laemmli (1970) and visualised by one of two methods:

a) Silver Staining

The method described by Wray et al. (1981) was followed exactly to stain low amounts of protein.

b) Coomassie Blue Staining

Gels were stained and fixed in 0.25% (w/v) Coomassie blue, 50% (v/v) methanol, 10% (v/v) acetic acid for 1 hour and destained in 40% (v/v) methanol, 10% (v/v) acetic acid to remove excess stain.

Gels were dried by vacuum desiccation at 80°C onto 3MM paper. To visualise radioactive proteins the gels were first flourographed by immersing in 'Amplify' for 15 min and after drying, autoradiographed by exposing to Fuji X X-ray film at -70°C.

2.13.2 Analysis of Ribosome Distributions.

Ribosome distributions were analysed on 11 ml 7-40% (w/v) concave exponential sucrose gradients (Noll, 1969) in the following

gradient buffer: 10 mM Tris/HCl, pH 7.5, 70 mM NH_4Cl and 4 mM $Mg(OAc)_2$. The gradients were made using a Densiflow II gradient maker (Buchler Instruments). Reticulocyte lysate translation mixtures were diluted 10-fold in ice-cold gradient buffer before loading onto the gradients. Gradients were centrifuged at 280,000g and 4°C for 110 min in a Beckman SW 41 rotor. Following centrifugation gradients were fractionated by upward displacement using an ISCO density fractionator and absorbance monitor (Instrumentation Specialities Co) and 0.5 ml fractions were collected. To ensure that [^{35}S] met-tRNAi bound to 40S subunits was detected, radioactivity in gradient fractions was determined after precipitation with 1% CTAB (Darnborough et al., 1972). To each fraction 0.5 ml 2% (w/v) CTAB in 20 mM NaOAc/acetic acid, pH 5.2 was added followed by 0.5 ml 0.5 M NaOAc/acetic acid, pH 5.2 containing 250 µg carrier yeast RNA. RNA was allowed to precipitate by incubating at 30°C for 10 min and the precipitates collected by filtration through Whatman GF/C filters. The filters were washed twice in sterile distilled water and dried under an infrared lamp. The radioactivity bound to each filter was estimated by counting in 4 ml LKB Optiphase Safe scintillation fluid in an LKB 1212 counter.

2.13.3 Dipeptide Formation.

Inhibited and control reticulocyte lysates were fractionated on sucrose gradients as described above (2.13.2) and the fractions containing 80S monosomes were pooled for each sample. Monosomes were pelleted at 100,000 rpm for 40 min at 2°C in a Beckman TL-100.3 rotor

and the pellets resuspended in 5 µl of 20 mM Tris/HCl, pH 7.5. An equal volume of 20 mM NaOH and 2 µl of 20 mM met-val dipeptide were added to each and the samples incubated for 15 min at 37°C to hydrolyse peptidyl-tRNA bonds (Crystal et al., 1974). The samples were applied directly to G1500 silica gel plates along with markers of methionine, valine and met-val, and developed in n-butanol/acetic acid/H_2O (12:3:5 (v/v/v)). Marker spots were visualised with 0.2% (w/v) ninhydrin in acetone and the positions of radioactive spots determined by autoradiography.

SECTION 2.14 PURIFICATION OF eEF-2 FROM WHEAT GERM.

2.14.1 Preparation of a High Speed Supernatant.

A high speed supernatant was used as the source for the subsequent purification of wheat germ eEF-2. The initial steps of the method of preparation from 50g of wheat germ was exactly as described for the purification of wheat germ ribosomes (section 2.3.3). To the pooled supernatants from the ultracentrifuge step solid ammonium sulphate was added up to 70% saturation, with constant stirring and left on ice for 1 hour. The precipitate was collected by centrifugation for 20 min at 18,000 rpm and 4°C in a Beckman 8x50 rotor and dissolved in 20 ml buffer A + 50 mM KCl (buffer A: 20 mM Tris/HCl, pH 7.6, 5% (v/v) glycerol, 3 mM β mercaptoethanol). This was dialysed overnight against 2 l of buffer A + 50 mM KCl.

2.14.2 DEAE-Cellulose Chromotography.

DEAE-cellulose was precycled and equilibrated with buffer A + 50 mM KCl, as recommended by the manufacturers, and a 1.5 cm x 30 cm column was prepared. The desalted protein was applied to the column and unbound protein eluted by washing the column in 50 ml of buffer A + 50 mM KCl. eEF-2 was eluted with buffer A + 300 mM KCl and collected in 5 ml fractions. Fractions containing eEF-2 were pooled (60 ml) and solid ammonium sulphate added up to 70% saturation with constant stirring. After 1 hour on ice the precipitate was collected by centrifugation (see section 2.14.1), dissolved in 10 ml of buffer A + 100 ml KCl and dialysed overnight against 2 l of the same buffer.

2.14.3 Cellulose Phosphate Chromotography.

A 1.5 cm x 25 cm column of cellulose phosphate was prepared and equilibrated, as recommended by the manufacturers. The dialysed fraction from the DEAE-cellulose step was loaded onto the column and the protein eluted by washing in buffer A + 100 mM KCl. The flow rate was maintained at 0.7 ml/min and 3 ml fractions were collected. Bound proteins were eluted with 30 ml buffer A + 350 mM KCl. eEF-2 was identified in fractions which came straight through the column and these fractions were pooled (30 ml). A 70% saturation ammonium sulphate precipitation was performed as described above, the precipitate dissolved in 4 ml buffer A + 100 mM KCl and dialysed overnight against 1 l of buffer A + 100 mM KCl.

2.14.4 Heparin Sepharose Chromotography.

The protein from the previous step was loaded onto a 0.9 cm x 15 cm heparin Sepharose column equilibrated in buffer A + 100 mM KCl. Unbound protein was washed through with 30 ml of buffer and eEF-2 eluted with buffer A + 200 mM KCl. Fractions with the highest activity were pooled and dialysed overnight against three changes of 1 l of 7.5 mM potassium phosphate buffer pH 8.0, 3 mM β mercaptoethanol.

2.14.5 Hydroxylapatite Chromotography.

The dialysed protein was loaded onto a 0.9 cm x 5 cm hydroxylapatite column, equilibrated in 7.5 mM potassium phosphate buffer pH 8.0, 3 mM β mercptoethanol and the column washed with 20 ml 100 mM potassium phosphate buffer pH 8.0, 3 mM β mercaptoethanol. eEF-2 was eluted with 0.5 M potassium phosphate buffer pH 8.0 and the fractions with the highest activity (4 ml) pooled and dialysed overnight against 1 l of 25 mM Hepes/KOH, pH 7.6, 60 mM KOAc, 2.5 mM $Mg(OAc)_2$, 5% (v/v) glycerol, 3 mM β mercaptoethanol. The purified eEF-2 contained in a dialysis bag was concentrated approximately 2-fold on aquacide and stored in 200 ul aliquots at -20°C.

2.14.6 eEF-2 Assay.

To identify fractions containing eEF-2 and to estimate the concentration of purified eEF-2, the method of Gill and Dinius (1971,

1973) was used. Purified diphtheria toxin was activated by trypsin cleavage (Gill and Dinius, 1971). To assay for eEF-2, 20 µl of each fraction was incubated at 37°C for 20 min in a final volume of 50 µl containing: 40 mM Tris/HCl, pH 8.0, 40 mM NH_4Cl, 40 µg BSA, 20 mM DTT, 2 µg activated diphtheria toxin and 50,000 cpm [^{14}C] NAD. TCA was added to a final concentration of 10% (w/v) and protein allowed to precipitate on ice for 10 min. Precipitates were collected on Whatman GF/C filters, washed and counted as described in section 2.13.2.

To estimate the concentration of eEF-2, the same assay was used. Samples were assayed in triplicate with and without the addition of diphtheria toxin. The concentration of eEF-2 was calculated from the difference in counts between the samples with and without toxin, assuming that all the eEF-2 is ribosylated with [^{14}C] NAD and that 1 mol eEF-2 binds 1 mol NAD(Gill and Dinius, 1973).

CHAPTER 3.

THE SITE OF ACTION OF RICIN A CHAIN ON EUKARYOTIC RIBOSOMES.

SECTION 3.1 INTRODUCTION.

In the pioneering work by Endo et al (1987) on rat 28S rRNA modification by ricin A chain, the site of action was deduced from direct RNA sequencing of a naturally occurring 550 nucleotide 3' terminal fragment. Furthermore, the authors showed that depurination of the rRNA at this position, A_{4324}, by ricin A chain could be assayed by the appearance of an approx. 390 nucleotide fragment produced by amine-catalysed cleavage of the 28S rRNA at this site. This elucidation of the mode of action of ricin A chain presented the opportunity to study the site of action of ricin A chain on other eukaryotic ribosomes and to investigate whether other ribosomes were also inactivated by the removal of the same adenine in the highly conserved sequence identified in rat 28S rRNA. However the approach used by Endo and coworkers of RNA sequencing on small naturally occurring fragments of the rRNA was not directly applicable to other ribosomes where comparable fragments were not known. Instead primer extension coupled with dideoxynucleotide sequencing was used. The later approach has been used extensively in structural probing of E.coli rRNA (Moazed et al., 1988, Stern et al., 1986). The rational for its use is based on the observation of Hagenbuchle et al. (1978) and Youvan and Hearst (1979) that reverse transcriptase is unable to read certain chemically modified bases in an RNA template. Pauses or stops give rise to bands corresponding to the length of the cDNA from the 5' end of the primer to the nucleotide immediately preceding the modified position. In the case of ricin A chain catalysed depurination of the rRNA it was envisaged that the removal of the

base would cause an additional band not present when untreated rRNA was used as the template. This approach has the advantage that it is less time consuming than direct RNA sequencing but it does rely on prior sequence knowledge to be able to design the primer.

Initially yeast ribosomes were targeted for study. The reasons for this were three fold; firstly the sequence of the LSU-rRNA was known both for Saccharomyces cerevisiae (Georgiev et al., 1981) and Saccharomyces carlsbergensis (Veldman et al., 1981). Secondly, more is known about the structure and function of the yeast cytoplasmic ribosome than that of any other eukaryote. Part of the reason for this progress is the well-defined genetics in yeast and the availability of mutants with altered ribosomal proteins (reviewed in Warner, 1982). Thirdly, other work in progress in our laboratory was aimed at obtaining yeast mutants with ricin-resistant ribosomes. The biochemical characterisation of the altered component(s) in such mutants could shed light on the action of ricin A chain. It was thought that all these would be of help if further investigation into the site of action of ricin A chain was carried out. On a practical note the isolation of intact ribosomes from yeast proved to be relatively unproblematic and little degradation of the rRNA is apparent in these ribosomes.

S.cerevisiae 26S rRNA is 3392 nucleotides in length, considerably shorter than rat 28S rRNA and it is almost identical to the corresponding LSU-rRNA from S.carlsbergensis. This is especially true in the 3' terminal region encompassing the highly conserved sequence identified by Endo - over the last 400 nucleotides both LSU-rRNAs are identical except for the addition of a single base in S.

carlsbergensis. This has the consequence that although the base in the conserved loop analogous to A_{4324} in rat 28S rRNA and A_{2661} in E.coli 23S rRNA in both yeasts is designated A_{3024}, it is 368 nucleotides and 369 nucleotides from the 3' end in S.cerevisiae and S.carlsbergensis respectively.

The site of action of ricin A chain was also investigated on plant ribosomes using wheat germ ribosomes as the system. Although no sequence for the entire 25S rRNA has been published, part of the 3' terminus has been deduced from sequence comparisons of the cloned intergenic region of wheat rDNA with other LSU-rRNAs (Barker et al., 1988). This region covers the ricin A chain / α-sarcin loop but because the entire sequence of the 25S rRNA is not known it is not possible to designate numbers to the nucleotides.

In these and subsequent experiments described in other chapters recombinant ricin A chain was used. The source of this is discussed in section 2.1.1. It differs from the native ricin A chain only in the presence of an additional residue (methionine) at it s N-terminus and the fact that it is non-glycosylated. The results of the following chapter show the native and recombinant proteins to be identical in activity and specificity.

SECTION 3.2 RESULTS AND DISCUSSION.

3.2.1 Effects of Ricin A Chain on Yeast Ribosomes.

Ricin A chain has been reported to inhibit protein synthesis in a number of eukaryotic cell-free translation systems, including

the yeast lysate (May, 1989). To test whether yeast ribosomes are modified by ricin A chain in a way analogous to that reported for rat 28S rRNA, ribosomes were purified from S.cerevisiae and incubated with and without ricin A chain for 1 hour. The rRNA was extracted and where indicated incubated with aniline to cleave at the site of depurination and the rRNA fractionated on 1.2% (w/v) agarose 50% formamide gels as described in section 2.6.1. (Fig. 3.2.1). The treatment of S.cerevisiae ribosomes with ricin A chain (tracks 3 and 4) or the incubation of extracted, untreated rRNA with aniline (tracks 5 and 6) does not cause the release of any additional fragments of rRNA when compared to the rRNA from control ribosomes (tracks 1 and 2). However when the yeast ribosomes are first incubated with ricin A chain and the extracted rRNA incubated with aniline a fragment is released (arrowed in tracks 7 and 8). To confirm that the fragment represents the 3' end of the 26S rRNA a probe to this sequence was generated from the EcoR1 fragment E of S. carlsbergensis rDNA (Veldman et al., 1981).

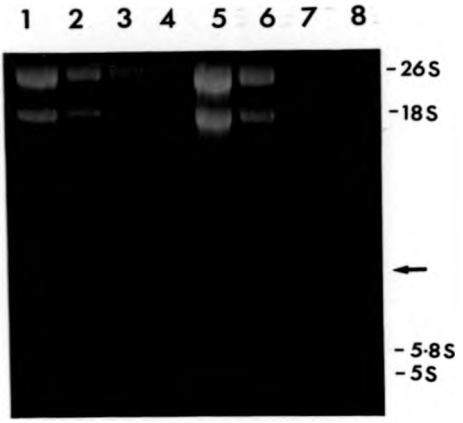
3.2.2 Generation of a Probe for the 3' end of 26S rRNA.

The EcoR1 fragment E of S.calsbergensis rDNA was a gift from Rudi Planta and contains 496 bp of the 3' terminus of the 26S rRNA gene and extends a further 100 bp into the spacer region (Fig. 3.2.2). Since this also contains approx. 125 bases upstream of the adenine analogous to the site of depurination in rat 28S rRNA a smaller probe was generated from the EcoR1 fragment E. The strategy for the cloning of this fragment is shown in Fig. 3.2.3. Briefly, the

Fig. 3.2.1 Gel electrophoresis of rRNA from S. cerevisiae ribosomes treated with ricin A chain.

Yeast ribosomes (50 ug) were incubated with 50 ng ricin A chain for 1 hour at 30°C in a final volume of 100 ul Endo buffer. A control, without ricin A chain was similarly incubated. The rRNA was extracted and where indicated treated with aniline as described in sections 2.4.1 and 2.5.1. The rRNA was fractionated on a 1.2% (w/v) agarose/ formamide gel and stained with EtBr (section 2.6.1).

1. control rRNA.
2. control rRNA.
3. rRNA from ribosomes incubated with ricin A chain.
4. rRNA from ribosomes incubated with ricin A chain.
5. aniline treated rRNA from control ribosomes.
6. aniline treated rRNA from control ribosomes.
7. aniline treated rRNA from ricin A chain treated ribosomes.
8. aniline treated rRNA from ricin A chain treated ribosomes.


1 2 3 4 5 6 7 8
-26S
-18S
-5·8S
-5S

```
   EcoR1
5' GAATTCGGTA AGCGTTGGAT TGTTCACCCA CTAATAGGGA
   ACATGAGCTG GGTTTAGACC GTCGTGAGAC AGGTTAGTTT
   TACCCTACTG ATGAATGTTA CCAGCAATAG TAATTGAACT
   TAGTACGAGA GGAACAGTTC ATTCGGATAA TTGGTTTTTG
   CGGCTGTCTG A CAGGCATT GCCGCGAAGC ACCATCCGCT
   GGATTATGGC TGAACGCCTC TAAGTCAGAA TCCATGCTAG
   AACGCGGTGA TTTCTTTGCT CCACACAATA TAGATGGATA
   CGAATAAGGC GTCCTTGTGG CGTCGCTGAA CCATAGCAGG
   CTAGCAACGG TGCACTTGGC GGAAAGGCCT TGGGTGCTTG
   CTGGCGAATT GCAATGTCAT TTTGCGTGGG GATAAATCAT
   TTGTATACGA CTTAGATGTA CAACGGGGTA TTGTAAGCGG
   TAGAGTAGCC TTGTTGTTAC GATCTGCTGA GATTAAGCCT
   TTGTTGTCTG ATTTGTTTTT TATTTCTTTC TAAGTGGGTA
   CTGGCAGGAG CCGGGGCCTA GTTTAGAGAG AAGTAGACTC
   AACAAGTCTC TATAAATTTT ATTTGTCTTA AGAATTC 3'
                                      EcoR1
```

Fig. 3.2.2 Sequence of the EcoR1 fragment E of S.carlsbergensis 26S rRNA.

Boxed is the Bcl 1 site used in the subcloning and circled is the putative site of action of ricin A chain.

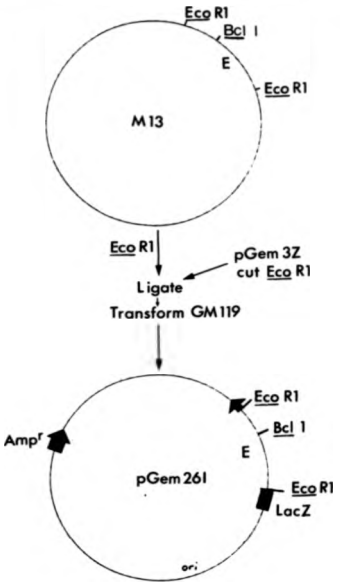


Fig. 3.2.3 Cloning strategy for the generation of the Bcl1/EcoR1 probe.

The Eco R1 fragment E was cut from replicative form M13 with Eco R1 and ligated into Eco R1 cut pGem 3Z. E.coli GM 119 was transformed with the ligation mix and recombinant plasmid isolated. The orientation of the insert, relative to the direction of transcription of lac Z (arrowed) was checked by restriction enzyme digests.

fragment E was inserted into the EcoR1 site of pGem-3Z (see Appendix 1) and cloned into E.coli GM 119. Purified recombinant plasmid was digested with EcoR1 and Bcl 1 and the larger 422 bp fragment was recovered after electrophoresis in a 1% agarose/TAE gel by electrophoresis onto a dialysis membrane as described in section 2.7.1. It was labelled to a specific activity of $4x10^7$ dpm/ug by nick-translation (section 2.9.1).

3.2.3 Northern Blotting and Hybridisation.

The rRNA from Fig. 3.2.1 was transfered to a Biodyne nylon membrane by capillary blotting and hybridised with the nick-translated Bcl 1-EcoR1 probe. The methods for this and the conditions for washing the membrane are described in section 2.10. Fig. 3.2.4 shows an autoradiograph of this Northern blot. In the untreated control samples and samples treated with A chain or aniline alone, the probe hybridised exclusively with the 26S rRNA (tracks 1-6). However in ricin-modified aniline-cleaved rRNA the probe hybridised only to the ca. 365 nucleotide fragment released (tracks 7 and 8) showing that the fragment is derived from the 3' end of the 26S rRNA.

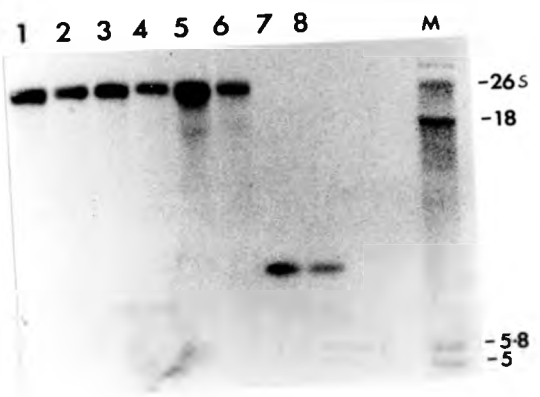
The observation that the probe hybridises solely to the ca. 365 nucleotide fragment and that there is no hybridisation to the intact 26S rRNA in these tracks shows that under conditions where the rRNA is completely modified at high concentrations of ricin A chain, the cleavage of the rRNA at this position is complete. Both the cleavage of the rRNA by aniline and the release of the fragment from the large 5' fragment under denaturing conditions must therefore also

Fig. 3.2.4 Autoradiograph of yeast rRNA probed with the nick-translated Bcl 1/EcoRl fragment.

The rRNA shown in Fig. 3.2.1 was transfered to a Biodyne nylon membrane by capillary blotting overnight. Northern hybridisation was carried out as described in section 2.10 and the membrane autoradiographed for 4 days next to Fuji X-ray film.

1. 4 µg control rRNA.
2. 2 µg control rRNA.
3. 4 µg rRNA from ribosomes incubated with ricin A chain.
4. 2 µg rRNA from ribosomes incubated with ricin A chain.
5. 4 µg aniline treated rRNA
6. 2 µg aniline treated rRNA.
7. 4 µg aniline treated, ricin A chain modified rRNA.
8. 2 µg aniline treated, ricin A chain modified rRNA.

M - ^{14}C labelled yeast rRNA.


1 2 3 4 5 6 7 8 M
-26S
-18
-5.8
-5

go to completion. Both these steps do not seem to be limiting which means that the percentage depurination can be calculated by measuring the amount of the fragment produced.

During this work Bradley et al. (1987) published the results of a similar investigation using S.cerevisiae ribosomes where the rRNA was probed with an oligodeoxyribonucleotide complementary to a conserved region near the 3' end of the 26S rRNA. These authors also showed that the probe hybridised to the fragment released by aniline following modification by ricin A chain. Both this observation and the results described above pointed towards the site of depurination in yeast 26S rRNA being in the same highly conserved loop identified by Endo and coworkers in rat 28S rRNA. To verify this conclusion the precise site of depurination was identified in a number of different eukaryotic ribosomes using primer extension.

3.2.4 Primer Extension.

The rational for using primer extension has been outlined above in the introduction. The primers used were ^{32}P, 5' end-labelled 17-mers complementary to a region of the rRNA approx. 70 nucleotides to the 3' side of the putative modification site. The sequence and position to which they anneal on the rRNA are shown in Fig. 3.2.5. Fig. 3.2.6 shows a sequencing gel of the products of primer extension reverse transcribed from unmodified yeast 26S rRNA (tracks 1-3), ricin A chain-modified-cleaved rRNA (track 4) and modified but uncleaved rRNA (track 5). The tracks labelled G,A,C and T are the products of primer extension on unmodified rRNA in the presence of

YEAST

5' CATAATCCAGCGGATGG 3'

3105 3089

WHEAT GERM

5' CATAATCCGGCTCACGG 3'

210 194

Fig. 3.2.5 Sequence of primers for reverse transcription on yeast and wheat germ rRNA.

The positions to which the primers anneal are denoted by the numbers below the 5' and 3' ends. For the yeast primer the numbers represent the bases in the 26S rRNA according to the sequence of Veldman et al. (1981). In the case of the primer used for wheat germ 25S rRNA because the entire sequence is not known, the numbers refer to the sequence for the 3' end contained within the intergenic region of wheat rDNA (Barker et al., 1988).

Fig. 3.2.6 Sequencing gel showing the products of primer extension on ricin A chain modified and unmodified yeast rRNA.

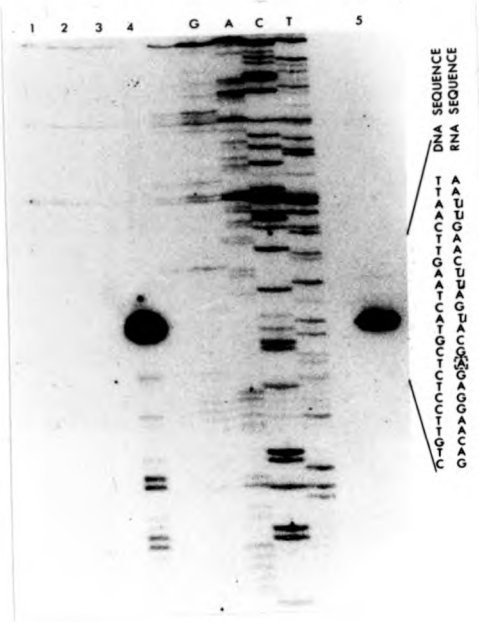
Yeast ribosomes were incubated with and without the addition of ricin A chain for 1 hour at 30 ^{o}C. The conditions were as described in Fig. 3.2.1. Total RNA was extracted (section 2.4.1) from the reaction mixtures and used as the template for reverse transcriptase. The conditions for annealing the primer and extension are shown in section 2.11.1. The products of these reactions were fractionated on a 8% polyacrylamide gel and visualised by autoradiography (section 2.6.3).

1-3. unmodified rRNA used as the template.

4. ricin A chain modified and aniline cleaved rRNA template.

5. ricin A chain modified rRNA template.

G,A,T and C refer to sequencing reactions carried out on the unmodified rRNA template in the presence of the respective dideoxynucleotide at a final concentration of 25 μM.


1 2 3 4 G A C T 5
DNA SEQUENCE
RNA SEQUENCE
TTAACTTGAATCATGCTCTCCTTGTC
AAUUGAACUUAGUACGAGAGGAACAG

the corresponding dideoxynucleoside triphosphate. When unmodified rRNA is used as a template for primer extension the result is a ladder of bands, corresponding to pauses or termination of the reverse transcriptase. However in both the modified-cleaved and the modified but uncleaved rRNAs there is a major termination site corresponding to G_{3025} in the 26S rRNA sequence, which is not present in the unmodified rRNA sample. Thus the site of depurination can be inferred from this as one base to the 5' of the termination site i.e A_{3024}.

The same approach was used to deduce the site of depurination in wheat germ 25S rRNA. Fig. 3.2.7 shows a sequencing gel of the products of primer extension using wheat germ 25S rRNA as the template. In the unmodified rRNA there is a strong band (track 4) which is also present in the toxin treated samples and which could represent a methylation site in the rRNA. However in addition there is another site which is only present when the rRNA has been modified by ricin A chain (track 1). This additional band corresponds to the second G in the conserved sequence GAGAGG and identifies the site of depurination as the first A in this sequence. This is the analogous base identified as the site of depurination in yeast 26S rRNA and by Endo in rat 28S rRNA.The site of action of the two type 1 RIPs from Dianthus caryophyllus, dianthin 32 and dianthin 30 was compared to ricin A chain. In wheat germ 25S rRNA both proteins have the same activity as ricin A chain (tracks 2 and 3).

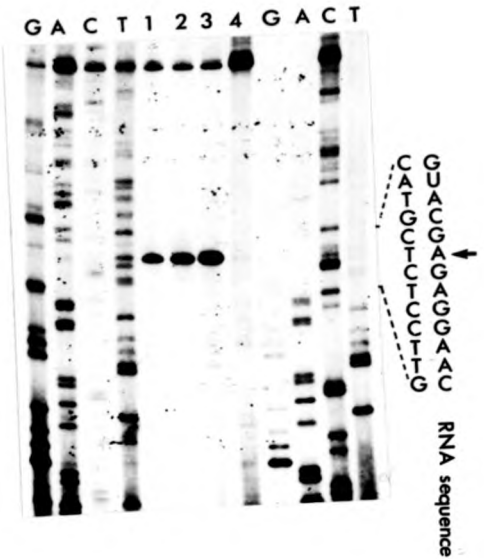
It appears likely that all sensitive ribosomes are modified by ricin A chain and other RIPs at the same base in the highly conserved sequence. The results of a similar investigation into the

Fig. 3.2.7 Sequencing gel showing the products of primer extension on unmodified and RIP modified wheat germ 25S rRNA.

Wheat germ ribosomes (72 µg) were incubated for 1 hour at 30 °C with either 10 µg ricin A chain, 0.1 µg dianthin 32, 0.1 µg dianthin 30 or without addition in a final volume of 100 µl wheat germ buffer. The RNA was extracted (section 2.4.1) and used as the template in the primer extension reactions (section 2.11.1). The products of the reactions were fractionated on a 8% polyacrylamide gel and visualised by autoradiography (section 2.6.3).

1. Ricin A chain modified template.
2. Dianthin 32 modified rRNA template.
3. Dianthin 30 modified rRNA template.
4. Unmodified rRNA template.

G,A,C and T are the products of sequencing reactions carried out on unmodified 25S rRNA in the presence of the appropriate dideoxynucleotide.


G A C T 1 2 3 4 G A C T
GUACGAGAGGAAC
CATGCTCTCCTTG
RNA sequence

site of depurination in rabbit 28S rRNA is published in May et al. (1989), but because these results are essentially the same as those described above they have not been presented here. In agreement with the results presented here Stirpe et al. (1988) identified A_{3024} in yeast 26S rRNA as the site of action of a number of type 1 RIPs. These authors used direct RNA sequencing to identify the missing base but the method of primer extension would seem to be a quicker and easier way of identifying the site of depurination, providing sequence data is available to design the primer. Unfortunately there is no universally conserved sequence downstream of the site of depurination in these large rRNAs that could be used as a primer and circumvent the need for sequence data if the action of ricin A chain was investigated on other eukaryotic ribosomes. The high degree of homology, however, in closely related LSU-rRNAs i.e. plant LSU-rRNAs, may mean that a universal primer could be designed to probe only these ribosomes.

CHAPTER 4.

RICIN A CHAIN CATALYSED INHIBITION OF PROTEIN SYNTHESIS.

SECTION 4.1 INTRODUCTION.

The elucidation of the mechanism of action of ricin A chain and the finding that it acted as a highly specific N-glycosidase answered the major question surrounding the action of this and related toxins. In addition the localisation of this site in a highly conserved sequence which had been implicated in E.coli 23S rRNA as the binding site for the elongation factors was in broad agreement with the large number of papers which have addressed the step of protein synthesis inhibited by ricin A chain. However the disagreement surrounding exactly which of these steps are inhibited remained unresolved. In the light of this the logical next step was to look at this whole question. In particular the question of whether initiation is also inhibited in addition to ricin A chain's well documented effect on peptide chain elongation.

The inhibition of elongation by ricin A chain has been studied in detail by a number of groups over the years and whilst there is agreement that it is inhibited, as discussed in the main introduction, there is no consensus on which particular step or steps within this cycle are inhibited. This is perhaps surprising since the same system of poly(U) - directed synthesis of polyphenylalanine was used in all the reports. The nature of these assays was designed to look solely at individual steps of elongation and because the poly(U) system does not rely on the normal pathway of initiation these authors were unable to show any additional effect on peptide chain initiation. Only one paper has addressed the question of whether initiation is also inhibited and these authors found that

in a wheat germ lysate the type 2 RIP abrin did inhibit initiation (Skorve et al., 1977). Abrin did not affect the binding of met-tRNAi and mRNA to the 40S subunit, but did interfere with the formation of the 80S initiation complex.

By using cell-free translation systems, in which natural mRNA can be translated, effects on initiation can be distinguished from those on elongation providing the system can be made initiation dependant. In the case of ricin A chain the use of the wheat germ lysate system was not possible because of the insensitivity of this system to inhibition by ricin A chain. It was therefore decided to investigate the possible inhibition of initiation by ricin A chain using either the yeast lysate or the rabbit reticulocyte lysate. These cell-free lysates also have the advantage over the poly(U) system in that the conditions resemble more closely the conditions in vivo. In particular the concentrations of K^+ and Mg^{++}, which affect the structure of the ribosome (Spirin et al., 1963 and reviewed in 1986), are more physiological. Some of the discrepancies that have arisen from the use of the poly(U)-directed phenylalanine assay to assess the ricin A chain catalysed inhibition of protein synthesis could be the result of the high level of Mg^{++} used in these assays (5-15 mM). This is because at high concentrations of Mg^{++} the inhibition of protein synthesis catalysed by ricin A chain has been shown to be overcome or reversed (Cawley et al., 1979, Skorve et al., 1977).

SECTION 4.2. RESULTS AND DISCUSSION.

4.2.1 Correlation of the Inhibition of Protein Synthesis with 28S rRNA Depurination.

Before investigating the steps of protein synthesis inhibited by ricin A chain preliminary experiments were undertaken to assess the effects of different amounts of ricin A chain on the incorporation of [^{35}S]-methionine into trichloroacetic acid insoluble product. Initially a yeast lysate translation system was used but because of difficulties in interpreting the effects on initiation and elongation, discussed in the following section, a reticulocyte lysate was used instead. Translation mixtures lacking label and mRNA were incubated with varying amounts of ricin A chain for 10 min. [^{35}S]-methionine and globin mRNA were then added and the incorporation of the label into proteins measured over a 10 min period. The results (Fig. 4.2.1) show that ricin A chain causes complete inhibition of protein synthesis at amounts equal to or greater than 2 ng. Under the conditions used, 2 ng ricin A chain corresponded to a molar ratio of ricin A chain/ribosome of 1:450. The remainder of the reaction mixtures were phenol/detergent extracted and aliquots containing RNA were treated with aniline to cleave at the depurination site. In the reactions containing 2 ng or greater of ricin A chain, the extracted rRNA showed extensive depurination as judged by the appearance of a fragment of approx. 390 nucleotides following aniline treatment (Fig. 4.2.1, tracks 2 and 3). Thus there is a positive correlation between inhibition of protein synthesis and

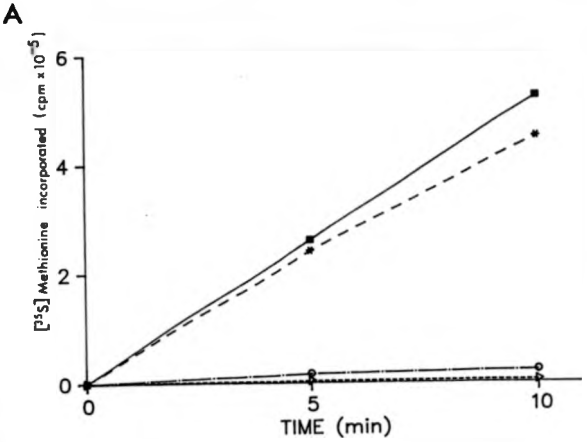
Fig. 4.2.1 Relationship between inhibition of protein synthesis and depurination of 28S rRNA by ricin A chain.

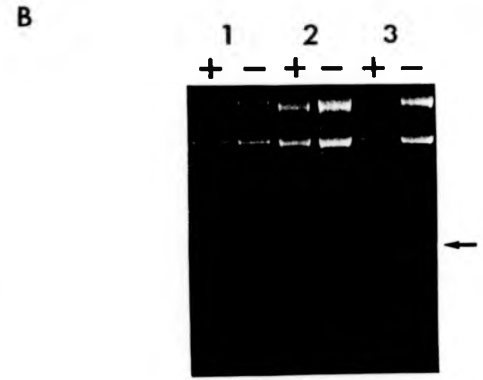
50 µl reticulocyte lysate reaction mixtures were incubated with the amounts of ricin A chain indicated (section 2.12.1 for reaction conditions). After 10 min 30 µCi [^{35}S] methionine and 0.125 µg globin mRNA were added and incubations continued for a further 10 min. (A) Trichloroacetic-acid-insoluble radioactivity was determined in duplicate 2 ul aliquots as described in section 2.12.1. Control (■——■); 0.2 ng ricin A chain (✱— - - —✱); 2 ng ricin A chain (○—·—○); 20 ng ricin A chain (△·····△).

(B) Total RNA was extracted from the remainder of the reaction mixtures shown in (A) and aliquots treated with aniline as described in sections 2.4.1 and 2.5.1. 3 µg of aniline-treated and untreated controls were fractionated in an agarose/formamide gel and stained with EtBr as described in section 2.6.1.

1. 0.2 ng ricin A chain.
2. 2 ng ricin A chain.
3. 20 ng ricin A chain.

(+) indicates aniline treatment; (-) non-treated controls. The approximate 390 nucleotide fragment is arrowed.


A
[35S] Methionine incorporated (cpm x 10-5)
6
4
2
0
0
5
10
TIME (min)


B
1
2
3
+ − + − + −

28S rRNA depurination in agreement with the observation of Endo et al. (1988d) on poly(U)-directed phenylalanine incorporation by rabbit reticulocyte ribosomes. A more detailed investigation into this correlation has been carried out in this laboratory using a yeast lysate translation system (May, 1989) and confirms this positive correlation, although at low levels of depurination the corresponding inhibition of protein synthesis was greater than expected. This probably reflects modification of one ribosome on a polysome which would result in the other ribosomes on the same mRNA backing up behind the inhibited one.

In subsequent experiments in which ricin A chain was used to inhibit protein synthesis 0.2 μg of toxin per reaction was used to insure complete inhibition. This is an amount approx. 100-fold greater than that required to give substantial inhibition of protein synthesis and depurination.

4.2.2 Dual Effects on Initiation and Elongation.

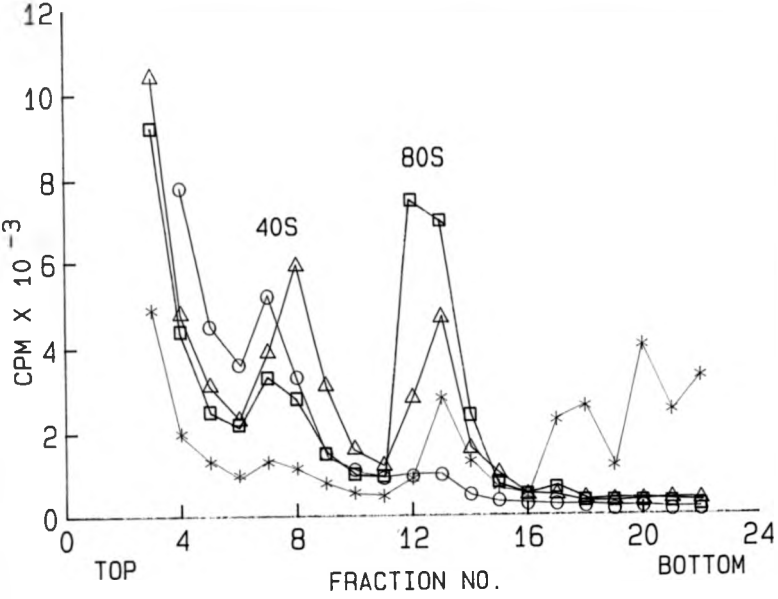
In order to distinguish between effects on initiation and elongation the cell-free translation system used needs to be mRNA dependant such that when mRNA is added there is an increase in the number of polysomes and monosomes reflecting initiation on the added mRNA. If the reaction mixture is preincubated with an inhibitor prior to the addition of the mRNA, the effect of that inhibitor can then be assessed on the subsequent initiation. Initially it was envisaged that a yeast lysate translation system would be used to assay for the effects of ricin A chain on initiation. This was in order to

complement previous work on yeast ribosomes, but it proved to be impossible to get sufficiently high levels of initiation to show an increase in the label associated with polysomes when mRNA was added. Even if the lysate was nuclease-treated the endogenous incorporation was high and little stimulation was seen when mRNA was added. This probably reflects the fact that the particular lysate was not very active rather than a general short-coming in this type of cell-free translation system since significant levels of stimulation have been reported using this and other yeast lysate systems (Rothblatt and Meyer, 1986, Tuite et al., 1980).

To get around these problems encountered with the yeast lysate a rabbit reticulocyte lysate was used instead. This system has the advantage that it has a high translational activity and that multiple rounds of protein synthesis occur on mRNAs (Palmiter, 1973). In this respect it is probably inherently better than the yeast lysate system. The rate of initiation was measured using the shift assay described by Darnbrough et al. (1973). The principle of this assay is that [^{35}S]met-tRNAi is first bound to the 43S preinitiation complex. If the 60S subunit subsequently joins the complex to form the 80S initiation complex then this is apparent on sucrose-density-gradients as a shift in radioactivity from the 40S to the 80S absorbance peaks. In the absence of elongation inhibitors, the radioactivity is subsequently found in the polysomes. If, on the other hand, elongation is inhibited, radioactivity accumulates in the 80S monosomes. Fig. 4.2.2 shows sucrose-density-gradient radioactivity profiles of control and inhibited lysates. In the control, the majority of the [^{35}S]met-tRNAi is associated with

Fig. 4.2.2 Sucrose gradient profiles of control and inhibited reticulocyte lysates.

Reticulocyte lysate reaction mixtures were incubated for 10 min with 1 μg diphtheria toxin and 7.5 nmol NAD (□———□); 0.2 μg ricin A chain (△———△); or without additions, (*———*) and (○———○). 0.25 μg globin mRNA and [^{35}S] Met-tRNAi (4 x 10^5 cpm) were added to each sample; aurintricarboxylic acid (5 mmol) was also added (○———○). The incubations were continued for a further 2 min and the samples fractionated on sucrose gradients as described in section 2.13.2. CTAB-precipitable radioactivity was determined in each 0.5 ml fraction (section 2.13.2).


12
10
8
6
4
2
0
CPM X 10 -3
40S
80S
0
4
8
12
16
20
24
TOP
BOTTOM
FRACTION NO.

polysomes. In contrast, incubation with aurintricarboxylic acid, which inhibits initiation by preventing the binding of mRNA to the 40S subunit (Huang and Grollman, 1972) results in [^{35}S]met-tRNAi labelling of only the 40S subunit. The profiles of the diphtheria toxin/NAD-treated and ricin A chain-treated lysates show labelling of the 40S subunit and the 80S monosome, but no labelling of the polysomes. This is consistent with their inhibitory effects on elongation. The concentrations of the inhibitors used were shown to be sufficient to inhibit protein synthesis completely (Fig. 4.2.3). A comparison of the ricin A chain inhibited and diphtheria toxin/NAD inhibited profiles shows that the 40S subunit labelling is greater in the former, but that the converse is seen with 80S monosomes. This suggests that the rate of formation of the 80S initiation complex may be lower in the ricin A chain-treated lysate than the diphtheria toxin-treated lysate.

To further investigate this possibility the ^{35}S-labelling of these complexes was compared after different time periods. Following a 1 min incubation (Fig. 4.2.4a) the differences in the relative labelling of the 40S subunit and the 80S monosomes are even more pronounced than described for those in Fig. 4.2.2. However, following a 10 min incubation (Fig. 4.2.4b) the differences are less pronounced. If ricin A chain acted solely on elongation, the rate of entry of ^{35}S into the 80S monosome would be expected to be comprable with that of the diphtheria toxin-treated lysate since this second toxin acts solely on elongation. That this is not the case is clearly demonstrated by comparing the ratio of [^{35}S]methionine-labelling of the 40S subunits and 80S monosomes following 1 min, 2 min and 10 min

Fig 4.2.3 Inhibition of protein synthesis by diphtheria toxin and aurintricarboxylic acid.

Reticulocyte lysate reaction mixtures were preincubated with 1 μg diphtheria toxin and 7.5 nmol NAD (■--------■) or without addition (○— — —○). Control reactions were similarly preincubated; (△———△) and (□———□). Globin mRNA and [^{35}S] methionine were added to each reaction; in addition 5 nmol aurintricarboxylic acid was also added (○ — — —○). The incubations were continued for a further 10 min. Trichloroacetic-acid-insoluble radioactivity was determined in 2 μl aliquots as described in section 2.12.1.

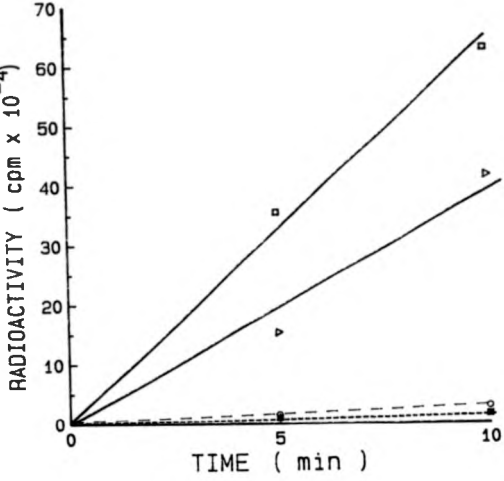
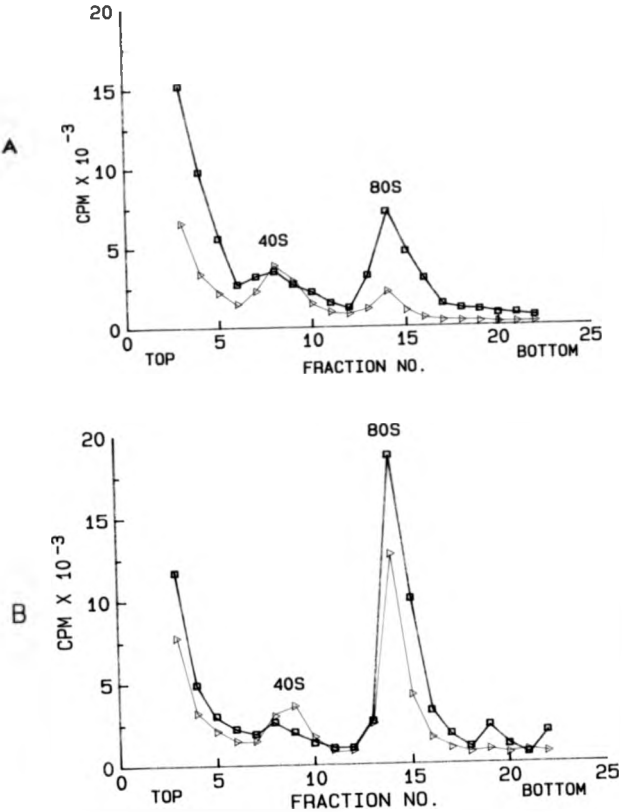

RADIOACTIVITY (cpm x 10^{-4})
70
60
50
40
30
20
10
0
0
5
10
TIME (min)

Fig. 4.2.4 Effect of time of incubation on the sucrose gradient profiles of inhibited lysates.

Reticulocyte lysates were incubated for 10 min with 0.2 μg ricin A chain (Δ———Δ) or 1 μg diphtheria toxin and 7.5 nmol NAD (□———□). Globin mRNA (0.25 μg) and [^{35}S] Met-tRNAi ($4x10^5$ cpm) were then added and the incubations continued for a further 1 min (A) or 10 min (B). The reactions were fractionated on sucrose-density gradients and the CTAB-precipitable radioactivity determined in each fraction as described in section 2.13.2.


A
CPM X 10 -3
20
15
10
5
0
80S
40S
0
5
10
15
20
25
TOP
BOTTOM
FRACTION NO.
B
CPM X 10-3
20
15
10
5
0
80S
40S
0
5
10
15
20
25
TOP
BOTTOM
FRACTION NO.

periods of ^{35}S labelling (Table 4.2.1). From this data, it is apparent that ricin A chain lowers the rate at which the 60S subunit joins the 48S preinitiation complex by a factor of about six.

This interpretation is supported by specific differences in the polysome profiles between the ricin A chain-treated lysates and the diphtheria toxin-treated or control lysates (Fig. 4.2.5). Inhibition of protein synthesis by ricin A chain causes an increase in the amount of free subunits and the appearance of half-mer polyribosomes. A similar profile has been reported in mutants of S. cerevisiae where depletion of the ribosomal protein L16 causes a decrease in 60S ribosomal subunits and a decrease in the rate of initiation (Rottenberg et al., 1988). In these mutants it was shown from the rRNA content of these peaks that the half-mer polyribosomes represented uncoupled 43S preinitiation complexes.

The reduction in the rate of initiation is consistent with the finding of Skorve et al. (1977) on the effect of abrin A chain on the wheat germ translation system. Since the only component of the translational apparatus to be affected by these toxins is the 60S subunit and the only involvement of the 60S subunit in initiation is the final step, this is most probably the step inhibited by ricin A chain.

4.2.3 The Steps Inhibited in the Elongation Cycle.

In agreement with previous work by other laboratories, the experiments described above show that in ricin A chain-treated lysates protein synthesis is arrested during the elongation cycle. To

Treatment	Duration of labelling (min)	Amount of radioactivity in 40S subunits and 80S monosomes (cpm)		Ratio 40S/80S
		40S	80S	
Ricin A chain	1	5853	3192	1:0.5
Diphtheria toxin	1	4937	14390	1:2.9
Ricin A chain	2	8538	8294	1:0.97
Diphtheria toxin	2	3329	15848	1:4.8
Ricin A chain	10	3723	18357	1:5
Diphtheria toxin	10	2144	30411	1:14.2

Table 4.2.1 Ratio of radioactivity in 40S subunits and 80S monosomes in the ricin A chain-inhibited and diphtheria toxin /NAD inhibited lysates incubated for varying times.

The amount of radioactivity in the 40S and 80S monsomes was calculated from the total radioactivity (cpm) in fractions 7-10 and 12-16 of Figs. 4.2.2 and 4.2.4, respectively and adjusted for a baseline of unbound ^{35}S sedimenting into the gradients.

Fig. 4.2.5 Absorbance profiles of inhibited and control lysates.

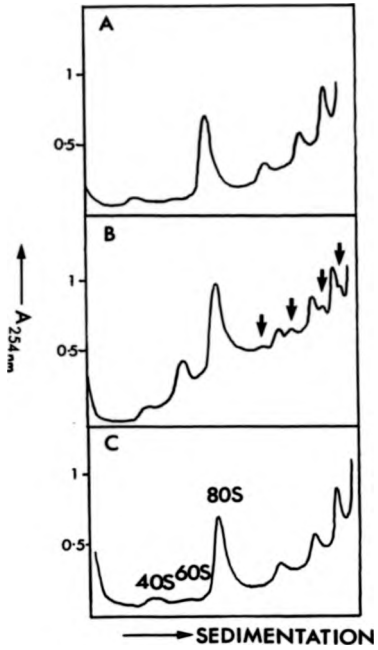
The experimental details and the fractionation on sucrose gradients was exactly as described in Fig. 4.2.2.

(A) Control.

(B) Ricin A chain inhibited.

(C) Diphtheria toxin / NAD inhibited.

The arrows indicate half-mer polyribosomes.


A
1
0·5
B
1
0·5
$A_{254\,nm}$
C
1
0·5
80S
40S
60S
SEDIMENTATION

test at which of the steps of this cycle the modified ribosomes had been inhibited, the peptides formed in these 80S monosomes following the initiation of protein synthesis using rabbit globin mRNA were analysed by thin layer chromotography. The rationale for this analysis is that the N-terminal dipeptide in both α and β globin chains is met-val (Fasman, 1976). Therefore in a nuclease-treated lysate, inhibition of the binding of the EF-1-GTP-Val-tRNA complex to the ribosome would result in only met-tRNAi being bound to 80S monosomes. On the other hand if peptidyl transferase was inhibited both met-tRNAi and val-tRNA would be bound, but not bonded together. Inhibition of translocation would result in formation of the met-val dipeptide.

In both the ricin A chain-inhibited and diphtheria toxin-inhibited lysates, inhibition results in the formation of predominantly the met-val dipeptide (Fig. 4.2.6). Methionine and two unidentified peptides of higher R_f than met-val were also present on these inhibited ribosomes but at much lesser amounts. On the other hand the peptides formed in the presence of the peptidyl transferase inhibitor anisomycin show a completely different pattern. In this case there are lesser amounts of met-val and at least four additional methionine-containing peptides not present in the other samples. The latter probably arise because anisomycin is known to give incomplete inhibition of protein synthesis (Battner and Vazquez, 1972) and thus they might represent longer peptides.

In the control incubations no discrete peptides were observed. This was possibly because the labelling of monosomes was low in the control, with most of the label associated with polysomes

Fig. 4.2.6 Peptides formed in the presence of ricin A chain, diphtheria toxin and anisomycin.

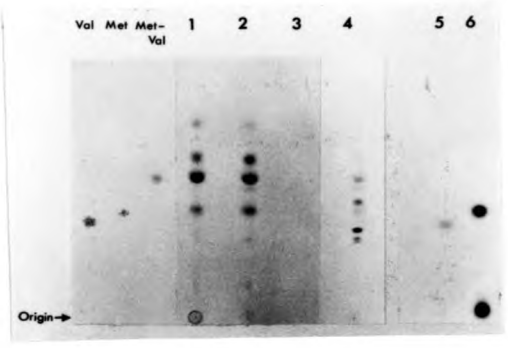
Micrococcal-nuclease-treated reticulocyte lysates were incubated with 0.2 μg ricin A chain, 1 μg diphtheria toxin and 7.5 nmol NAD, 12.5 nmol anisomycin or without addition for 10 min. Globin mRNA (0.25 μg) and [^{35}S] Met-tRNAi ($4x10^5$ cpm) were added to each reaction and the incubations continued for a further 5 min. The reaction mixtures were fractionated on sucrose gradients as described in section 2.13.2. The 80S monosome fractions were pooled for each sample, the ribosomes pelleted and ribosome-bound [^{35}S] Met and [^{35}S] Met-containing peptides released by alkali treatment as described in section 2.13.3. The peptides were then analysed by thin-layer chromotography and visualised by autoradiography (section 2.13.3).

Tracks 1,2,3 and 4 represent peptides released from diphtheria toxin and NAD-treated lysate, ricin A chain-treated lysate, control lysate and anisomycin-treated lysate, respectively.

5. ^{14}C-Val marker.

6. Alkali hydrolysis of [^{35}S] Met-tRNAi.

Met, Val and Met-Val represent ninhydrin-stained markers.


Val
Met
Met–Val
1
2
3
4
5
6
Origin→

(Fig. 4.2.2). At the same time chain heterogeneity would also be expected. Similar experiments incubated with ^{14}C labelled val-tRNA instead of labelled met-tRNAi failed to show any spots on the thin layer chromotography plate under any of the inhibited conditions. This was probably because it proved impossible to label the tRNA to a high enough specific activity to ensure the low levels of peptides were visible on the autoradiograph. Under the same conditions used in the assay, hydrolysis of met-tRNAi gave two spots, one of which had the same R_f as ninhydrin-stained methionine (Fig 4.2.6). The other one of lower R_f probably represents unhydrolysed [^{35}S]met-tRNAi.

The similarity in the distribution of [^{35}S] methionine-labelled peptides in the ricin A chain-treated and diphtheria toxin-treated lysates, and the predominance of met-val suggests both inhibitors block the elongation cycle at the translocation step. This is supported by the finding that anisomycin gives an entirely different pattern of methionine-containing peptides bound to the 80S monosomes. Nilsson and Nygard (1986) used a similar approach and also found a predominance of met-val, although their conclusions were limited, because no comparisons to other inhibitors were made. These authors also found relatively more methionine bound to the ribosomes in the ricin A chain-treated lysates than reported here. This possibly was a consequence of their analysis of the total ribosome pellet which would be expected to contain methionine bound to 43S preinitiation complexes.

4.2.4 Reactivity of Ricin A Chain Inhibited Ribosomes with Puromycin.

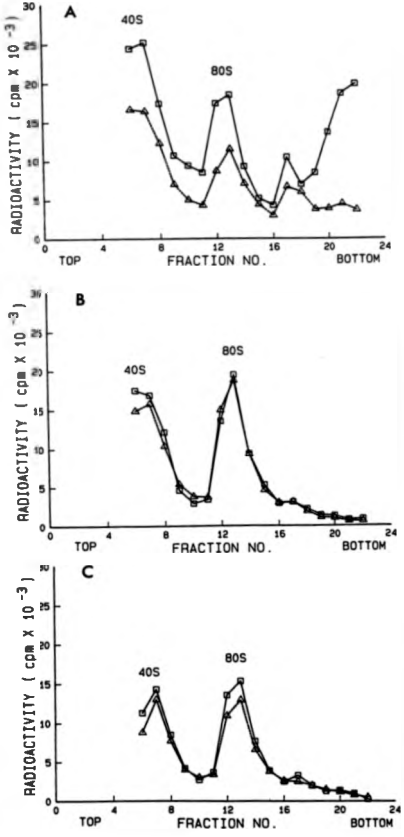
A prediction arising from the conclusion drawn above is that in ricin A chain inhibited and diphtheria toxin inhibited lysates, peptidyl-tRNA should occupy the ribosomal A site, which would therefore be blocked for the entry of puromycin. Although the formation of peptidyl-puromycin requires just the peptidyl transferase activity of the ribosome, the A site needs to be empty for the inhibitor to bind.

To test this prediction, reticulocyte lysates were incubated with ricin A chain, diphtheria toxin or without an inhibitor. [35S]methionine and globin mRNA were then added and incubations continued for a further 10 min. The reactions were then divided and puromycin added to one half of each, followed by a further 5 min incubation. [^{35}S]methionine was used instead of [^{35}S]met-tRNAi to ensure a high level of labelling in the monosome and polysome regions of the control, uninhibited lysate. Sucrose gradient profiles of these reaction mixtures are shown in Fig. 4.2.7. In the control incubation (Fig. 4.2.7a), puromycin causes most of the ^{35}S to be removed from monosomes and polysomes. In contrast, in both the diphtheria toxin-treated and the ricin A chain-treated lysates the majority of the ^{35}S bound to monosomes is unreactive with puromycin (Fig. 4.2.7b and c).

Essentially the same result was obtained using ricin A chain inhibited yeast polysomes. A yeast lysate translation mix was preincubated for 2 min before the addition of ricin A chain, and the incubation continued for 10 min. A control lysate was similarly

Fig. 4.2.7 Effect of ricin A chain and diphtheria toxin on the release of nascent peptides by puromycin.

0.1 ml nuclease-treated reticulocyte lysate reaction mixtures were incubated for 10 min (A) without addition, (B) with 2 μg diphtheria toxin and 15 nmoles NAD, or (C) with 0.4 μg ricin A chain. Globin mRNA (0.5 μg) and [^{35}S] methionine (25 μCi) were then added and incubation continued for a further 10 min. Reaction mixtures were then divided equally and 1 μl 10 mM puromycin added to one half, followed by incubation for 5 min. Samples were fractionated on sucrose gradients and CTAB-precipitable radioactivity was determined in each 0.5 ml fraction as described in section 2.13.2. Controls: without puromycin (□———□); with puromycin (Δ------Δ).


A
40S
80S
RADIOACTIVITY (cpm X 10 -3)
TOP
FRACTION NO.
BOTTOM
B
40S
80S
RADIOACTIVITY (cpm X 10 -3)
TOP
FRACTION NO.
BOTTOM
C
40S
80S
RADIOACTIVITY (cpm X 10 -3)
TOP
FRACTION NO.
BOTTOM

incubated but without toxin. Ribosomes were pelleted from each reaction through a sucrose cushion, resuspended and divided into two halves. One half of each was incubated with puromycin for 10 min, and the other half incubated but without addition. The ribosomes were re-pelleted and aliquots of the supernatants and the resuspended pellets were spotted onto filters and the TCA-insoluble radioactivity counted. The amount of radioactivity found in each of the fractions from the two reactions is shown in Table 4.2.2. In the control incubated without puromycin the majority of the radioactivity was bound to the ribosomes as expected. However following incubation with puromycin, a significant amount of the label is released from the ribosomes. In the ricin A chain inhibited lysate, there is a much smaller shift in label from the ribosomes to the supernatant when incubated with puromycin.

All three of the experiments that have addressed the position at which elongation is arrested by ricin A chain support the notion that it is the eEF-2 catalysed translocation step that is inhibited. However, from these results it is still possible that the binding of aminoacyl-tRNA is also inhibited to a lesser extent because even greatly reduced rates of binding, over a long incubation period would still result in met-val formation and unreactivity with puromycin if translocation was severely inhibited. There is however no way of reconciling these results with recent publications on the activity of the related Vero and Shiga toxins (Igarashi et al., 1987). These authors found that the binding of aminoacyl-tRNA was inhibited by these toxins in rabbit reticulocyte ribosomes, but that there was no effect on translocation.

Treatment	Puromycin	Radioactivity (cpm) (^{35}S)	
		Supernatant	Pellet
Control	-	3425	18750
Control	+	8234	5346
Ricin A chain	-	1150	11317
Ricin A chain	+	3425	8634

Table 4.2.2 Effect of ricin A chain on the release of nascent polypeptides by puromycin in a yeast lysate.

Yeast lysate reactions (100 µl) were incubated with ^{35}S methionine at 25 ^{o}C for 2 min. 4 µg ricin A chain was added and the incubations continued for a further 10 min. A control was similarly incubated. The reactions were diluted by the addition of 400 µl yeast ribosome buffer and the ribosomes pelleted by centrifugation through a 1 ml 1 M sucrose cushion for 30 min at 100,000 rpm in a Beckman TL-100.3 rotor. The ribosome pellets were resuspended in 50 µl yeast ribosome buffer and divided equally into two tubes. To one, 5 µl 1.2 mM puromycin was added, to the other, 5 µl distilled water. The reactions were incubated for 10 min at 25 ^{o}C, diluted to 200 µl with yeast ribosome buffer and the ribosomes re-pelleted. 2x 20 µl aliquots of the supernatants were spotted onto Whatman 3MM paper and TCA-insoluble radioactivity measured. The ribosome pellets were resuspended in 50 µl yeast ribosome buffer and 20 µl aliquots counted in duplicate as for the supernatant samples. The radioactivity in each fraction of the samples was calculated as an average.

CHAPTER 5.

THE DIFFERENTIAL SENSITIVITY OF RIBOSOMES FROM YEAST, WHEAT GERM AND RABBIT TO RICIN A CHAIN CATALYSED DEPURINATION.

SECTION 5.1 INTRODUCTION.

The preceding two chapters have presented results on the site of action of ricin A chain on ribosomes from different eukaryotic sources, and on the subsequent steps of protein synthesis which are inhibited as a result of this action. The similarity in the mode of action of ricin A chain against these different substrates raises the question of their relative sensitivity to modification and the factors which affect it.

In the past there have been a small number of papers published where the authors have compared the ability of ricin A chain to inhibit poly(U) translation on ribosomes from different sources. Cawley et al. (1977 and 1979) compared the sensitivity of ribosomes from wheat germ and rat liver to inhibition by ricin A chain. However the results published by these authors in the two papers differed. In the first instance ricin A chain was found to be unable to inhibit protein synthesis on wheat germ ribosomes at a concentration 5000 times higher than that which was effective against rat liver ribosomes. Whereas at the lower Mg^{++} concentration used in the second paper (10mM compared to 15mM), it was found that ricin A chain could inhibit protein synthesis on wheat germ ribosomes, but at a concentration only approximately 100 times higher than that was required to give the same inhibition in rat liver ribosomes. In addition Harley and Beevers (1982) reported that the concentration of ricin A chain required to give 50% inhibition in the same poly(U) translation system was approximately 40 µg/ml for wheat germ ribosomes compared to 1.6 ng/ml for those from rat liver, a

difference of approximately 20,000.

It is clear from these conflicting reports that the inhibition of poly(U) translation systems is not a reliable method of comparing the sensitivity of different ribosomes to inactivation by ricin A chain. One possible explanation is the elevated levels of Mg^{++} needed in these assays to force the translation on the poly(U) and the already discussed effects this has on the level of ricin A chain catalysed inhibition of protein synthesis. However by using the aniline assay, first descibed by Endo et al. (1987) and used in the preceding chapters, the sensitivity of different ribosomes can be compared at more physiological concentrations of monovalent and divalent cations. In addition this has the advantage that it measures the N-glycosidase activity of ricin A chain directly.

SECTION 5.2 RESULTS AND DISCUSSION.

5.2.1 Differential Sensitivity of Three Different Eukaryotic Ribosomes.

To test the sensitivity of ribosomes from wheat germ, yeast and rabbit reticulocytes to depurination by ricin A chain 30 µg of purified ribosomes were incubated for 20 min with different amounts of ricin A chain. The rRNA was extracted and incubated with aniline to cleave at the site of depurination before it was fractionated on agarose/formamide gels. With rabbit reticulocyte ribosomes there is substantial depurination, as judged by the appearance of the approx. 390 nucleotide fragment, at amounts of ricin A chain equal or greater

than 0.75 ng (Fig. 5.2.1). In comparison similar levels of depurination are only apparent when yeast and wheat germ ribosomes are incubated with 7.5 ng (Fig. 5.2.2) and 750 ng (Fig. 5.2.3) respectively. Incubation of the ribosomes from the three sources with amounts of ricin A chain 2-fold below these respective values gave no significant depurination over this time period. Under these conditions, rabbit reticulocyte ribosomes are approx. 10 and 1000 times more sensitive to ricin A chain catalysed depurination than yeast and wheat germ ribosomes respectively. In the case of the rabbit ribosomes, in this assay 0.75 ng represents a toxin/ribosome ratio of 1:300.

Since the conditions used in each assay were identical, the question arises as to the nature of these large differences in sensitivity. In particular whether the differences are due to inherent differences in the ribosomes or because of the presence or absence of supernatant factors in the crude ribosome preparations. It is possible that these differences are the consequence of protease activity in the different ribosome preparations. However it has been shown previously in this laboratory that ricin A chain mRNA is translated efficiently in a wheat germ lysate, but that in a reticulocyte lysate or a yeast lysate the translated protein quickly inhibits protein synthesis and little product is visible (May et al., 1989). In the wheat germ undegraded ricin A chain was found to accumulate. Thus, it is unlikely that protease activity in the ribosome preparations is responsible for the differences in sensitivity. On the other hand it has been reported that the level of inhibition of protein synthesis by ricin A chain is dependant on the

Fig. 5.2.1 The sensitivity of rabbit reticulocyte ribosomes to depurination by ricin A chain.

30 µg crude (tracks 1-3) and salt washed (tracks 4-6) ribosomes were incubated with the amounts of ricin A chain indicated below for 20 min at 30 °C in a final volume of 100 µl Endo buffer. A control without ricin A chain was similarly incubated (track -). Total RNA was extracted, 3 µg treated with aniline and the rRNA fractionated in an agarose/formamide gel as described in sections 2.4.1, 2.5.1 and 2.6.1.

1 and 4, 0.75 ng ricin A chain.

2 and 5, 0.375 ng ricin A chain.

3 and 6, 75 pg ricin A chain.

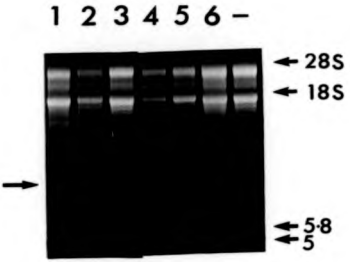

1 2 3 4 5 6 –
28S
18S
5·8
5

Fig. 5.2.2 The sensitivity of yeast ribosomes to depurination by ricin A chain.

30 µg crude (tracks 1-5) and salt washed (tracks 6-10) ribosomes were incubated for 20 min at 30 °C in a final volume of 100 µl Endo buffer with the amounts of ricin A chain indicated below. A control without ricin A chain was similarly incubated (track -). Total RNA was extracted, treated with aniline (3 µg) and run on an agarose/formamide gel as described in sections 2.4.1, 2.5.1 and 2.6.1.

1 and 6, 7.5 ng ricin A chain.

2 and 7, 3.75 ng ricin A chain.

3 and 8, 0.75 ng ricin A chain.

4 and 9, 0.375 ng ricin A chain.

5 and 10, 75 pg ricin A chain.

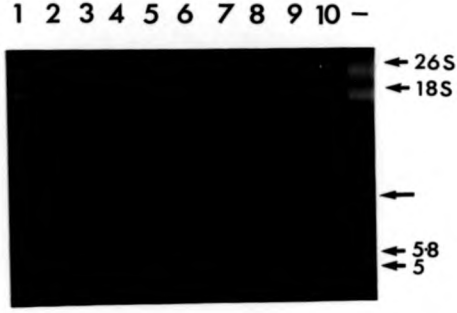

1 2 3 4 5 6 7 8 9 10 –
26S
18S
5S
5

Fig. 5.2.3 The sensitivity of wheat germ ribosomes to depurination by ricin A chain.

30 µg crude (tracks 1-6) and salt washed (tracks 7-12) ribosomes were incubated under the same conditions as used in Figs. 5.2.1 and 5.2.2 with the amounts of ricin A chain indicated below. A control without ricin A chain was also incubated (track -). Total RNA was extracted, 3 µg treated with aniline and the rRNA fractionated on an agarose/formamide gel as described in sections 2.4.1, 2.5.1 and 2.6.1.

1 and 7, 7.5 µg ricin A chain.

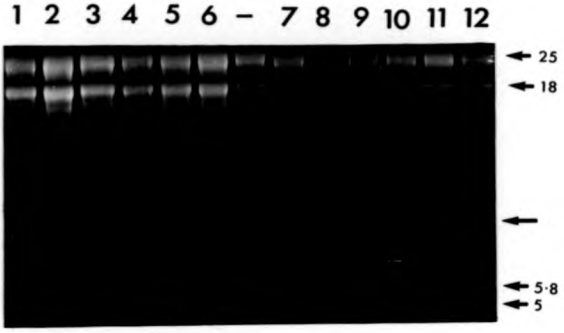
2 and 8, 750 ng ricin A chain.

3 and 9, 375 ng ricin A chain.

4 and 10, 75 ng ricin A chain.

5 and 11, 37.5 ng ricin A chain.

6 and 12, 7.5 ng ricin a chain.


1 2 3 4 5 6 – 7 8 9 10 11 12
25
18
5·8
5

amount of either one or both of the two elongation factors (Olsnes et al., 1975b, Fernandez-Puentes et al., 1976a). To investigate whether the differences outlined above could be the result of differences in the ribosomal content of these factors the ribosomes were salt-washed in 0.5 M KCl prior to incubation with ricin A chain. This treatment has been shown to remove bound elongation factors and aminoacyl-tRNA (Moldave and Skogerson, 1967, Crystal et al., 1974), without causing the complete dissociation of ribosmes engaged in translation into the constituent subunits that results from incubation at high salt in the presence of puromycin (Blobel, 1971). Ribosomes were incubated in 0.5 M KCl for 1 hr on ice, pelleted and washed and resuspended in the same low salt buffer used in the above experiments.

With the rabbit reticulocyte ribosomes there is a slight increase in sensitivity to ricin A chain-catalysed depurination when washed ribosomes are compared to the non-washed, native ribosomes, as judged by the appearance of the ca. 390 nucleotide fragment at lower amounts of ricin A chain (Fig. 5.2.1). There is also an increase in sensitivity when washed and non-washed ribosomes from yeast (Fig. 5.2.2) and wheat germ (Fig. 5.2.3) are compared. However in the later two cases this increase in sensitivity following salt washing is greater; the same approximate level of depurination found in the unwashed ribosomes is seen when salt-washed ribosomes are incubated with 10-20 times lower amounts of ricin A chain. At the same time, however, there are still differences in the sensitivity of the salt-washed ribosomes from the three different sources. So whilst the removal of a bound factor or factors increases the susceptibility

of these ribosomes to depurination, there are inherent differences in the ability of the different ribosomes to act as a substrate for the reaction. The possible reason for these differences are discussed in chapter 7.

5.2.2 Removal of Bound eEF-2 and Increase in Sensitivity.

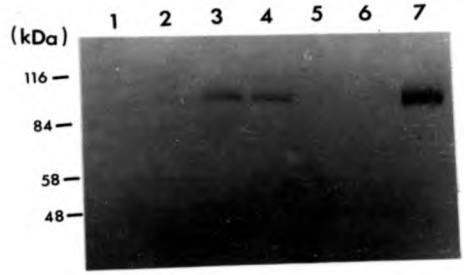
The amount of non-ribosome bound eEF-2 present in a given solution can be assayed by exploiting the activity of diphtheria toxin in uniquely ribosylating eEF-2 (Gill and Dinius, 1971, 1973). In the presence of ^{14}C-NAD, eEF-2 is specifically labelled and under defined conditions the amount of label incorporated is a reflection of the amount of eEF-2 present. The conditions for the assay are given in section 2.14.6.

The supernatants from salt-washed ribosomes, containing the protein and tRNA removed by the high salt, were assayed for the presence of eEF-2 and the proteins analysed by polyacrylamide gel electrophoresis (Fig. 5.2.4). In both the supernatants from the washed yeast and wheat germ ribosomes, the presence of a radioactive band of approximately 100,000 Da shows that an amount of eEF-2 has been removed from the ribosomes. In contrast little or no eEF-2 is present in the washed fraction from the rabbit reticulocyte ribosomes. There is no visible amount of eEF-2 in the supernatants from the low salt-washed ribosomes. Thus there is a correlation between the amount of eEF-2 removed by the high salt and the increase in sensitivity to depurination. The possibility that eEF-2 is protecting these ribosomes from ricin A chain catalysed depurination

Fig. 5.2.4 PAGE of fractions removed by high or low salt from yeast, rabbit reticulocyte and wheat germ ribosomes.

Ribosomes from the three sources (400 µg) were salt-washed as described in section 2.3.4. The same amount of each of the ribosomes was also washed in Endo buffer. The supernatants (0.8 ml) were removed from the pelleted ribosomes and dialysed overnight against 100 ml of Endo buffer. The volumes were corrected to 1 ml with Endo buffer and 20 µl aliqouts assayed for eEF-2 by incubating with diphtheria toxin and ^{14}C NAD in a final volume of 50 µl as described in section 2.14.6. 20 ul of wheat germ lysate was also assayed for eEF-2 to act as a marker. The protein in each sample was analysed on a 10% SDS polyacrylamide gel (section 2.13.1) and the ^{14}C labelled eEF-2 visualised by autoradiography.

1. Supernatant from Endo buffer washed reticulocyte ribosomes.
2. Supernatant from high salt washed reticulocyte ribosomes.
3. Supernatant from high salt washed wheat germ ribosomes.
4. Supernatant from high salt washed yeast ribosomes.
5. Supernatant from wheat germ ribosomes washed in Endo buffer.
6. Supernatant from yeast ribosomes washed in Endo buffer.
7. Wheat germ lysate.


(kDa)
1
2
3
4
5
6
7
116
84
58
48

and that its removal accounts for the increase in sensitivity is discussed further in the following chapter.

Zamboni *et al.* (1989) have recently shown that pretreatment of ribosomes with KCl and puromycin causes the release of more adenine molecules, as assayed for by FPLC, per molecule of ribosomes when they are then treated with ricin A chain. These authors found that the adenine released was often greater than 1 pmol/pmol of ribosomes and they concluded that the pretreatment exposed on the ribosomes one or more additional sites that became substrates for the RIP. However in the experiments described above, no additional rRNA fragments were seen when the salt-washed ribosomes were incubated with ricin A chain and the rRNA subsequently cleaved with aniline. These would be expected if the additional sites were exposed and became modified.

CHAPTER 6.

THE PROTECTION OF SALT WASHED RIBOSOMES FROM DEPURINATION BY RICIN A CHAIN.

SECTION 6.1 INTRODUCTION.

It has been reported that, in the presence of high levels of eEF-2, the ricin A chain-catalysed inhibition of protein synthesis is reduced (Fernandez-Puentes et al., 1976b, Brigotti et al., 1989). These authors have postulated that eEF-2 and ricin A chain share a common binding site on the ribosome and that their binding is mutally exclusive. There is however no direct evidence that either of the two elongation factors reduce the level of ricin A chain-catalysed modification in these assays. Other authors have explained the same results on the basis that the high levels of elongation factors are able to overcome the inhibitory action of ricin A chain on protein synthesis (Olsnes et al., 1975b). Support for the first idea has come from studies on the effect of eEF-2 on the binding of ricin A chain. Cawley et al. (1979) found that eEF-2, bound irreversibly to the ribosome with a non-hydrolysable analogue of GTP, could prevent labelled ricin A chain from binding to the ribosome. In this laboratory it was shown that ricin A chain and eEF-2 from hamster shared an homology of six amino acid residues (Asp,Val,Thr,Asn,Ala, Tyr) and that the deletion of this region from ricin A chain resulted in the loss of activity (May et al., 1989). Sequence homology in regions important for activity (i.e. binding) might be expected between proteins that interact with a common substrate in a similar way and this result has also been taken as evidence that ricin A chain and eEF-2 share some feature in the way they interact with the ribosome, but with the reservations discussed previously in section 1.7.2.

In the previous chapter it was shown that pre-treatment of ribosomes with high concentrations of KCl resulted in the ribosomes becoming more sensitive to depurination by ricin A chain. Furthermore where this treatment resulted in a large increase in sensitivity, as it did with the yeast and wheat germ ribosomes, there was a positive correlation between this and the amount of eEF-2 removed by the high salt. This observation would also seem to be in agreement with the idea that eEF-2 is able to protect ribosomes from the action of ricin A chain.

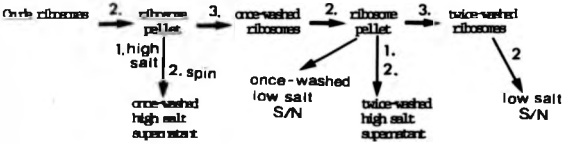
Treatment of ribosomes with high concentrations of KCl or NH_4Cl has been shown to remove many loosely bound protein synthesis factors (Moldave and Skogerson, 1967) and is often used to prepare vacant ribosomes which do not have these factors or aminoacyl-tRNA bound to them. This treatment does not however remove bona fide ribosomal proteins and in fact it is used as one of the distinguishing features between ribosomal proteins and other proteins which are found associated with the ribosome (Itoh et al., 1968, Lerman et al., 1966). The following sections of this chapter describe experiments carried out to investigate the ability of proteins loosely bound to the ribosome to protect ribosomes from ricin A chain-catalysed depurination. In particular the experiments address the question of whether eEF-2 bound to the ribosome directly lowers the level of ricin A chain-catalysed depurination. Wheat germ ribosomes were used in these experiments because of the large increase in sensitivity seen after salt-washing and because wheat germ proved to be a good system for the purification of eEF-2.

SECTION 6.2 RESULTS AND DISCUSSION.

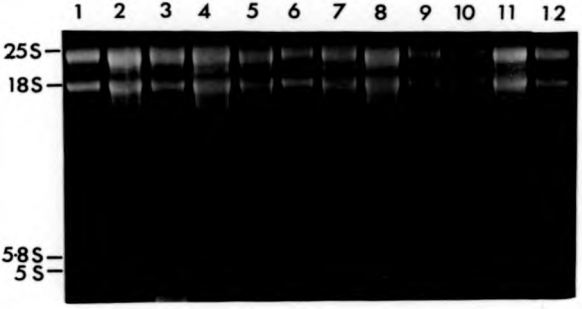
A prediction of the notion put forward in the last chapter that the increase in sensitivity of ribosomes to ricin A chain following salt-washing is due to the removal of a loosely bound protein, is that adding back the same protein should protect the ribosomes from the subsequent action of ricin A chain. Since treatment of ribosomes with 0.5 M KCl does not remove bona fide ribosomal proteins, the factor responsible for the protection might also be expected to be present in the post-ribosomal supernatant. To test this, salt-washed ribosomes were preincubated with and without the addition of wheat germ S-100 in the presence of GMPPCP. Wheat germ S-100 is the supernatant from a wheat germ lysate when the ribosomes are removed by centrifugation and so would be expected to contain all the supernatant proteins, including unbound protein synthesis factors. Following this 10 min incubation ricin A chain was added and the incubation continued for a further 15 min. The rRNA was extracted, treated with aniline and the rRNA fractionated on agarose/formamide gels. The level of depurination was estimated from the amount of the ca. 360 nucleotide fragment released by aniline. It can be seen that there is substantial depurination in the salt-washed ribosomes preincubated without the S-100 (Fig. 6.2.1, track 2). In contrast, preincubation with the S-100 protects the ribosomes from depurination since the aniline fragment is no longer prominent (track 3). This protection is also apparent when the washed ribosomes are preincubated with the dialysed supernatant from the treatment of ribosomes with high salt (track 4). In both cases the level of depurination in the protected ribosomes is

Fig. 6.2.1 Protection of salt washed ribosomes from depurination by ricin A chain.

36 µg crude or salt washed wheat germ ribosomes were incubated for 10 min at 30 °C with 0.1 µmol GMPPCP or where indicated with 0.1 µmol GMPPCP and fractions removed from wheat germ ribosomes by high and low salt in a total volume of 100 µl wheat germ buffer. 75 ng ricin A chain was added to each and the incubations continued for a further 15 min. The total RNA was extracted, 3 µg treated with aniline and the rRNA fractionated in an agarose/ formamide gel as described in sections 2.4.1, 2.5.1 and 2.6.1. In order to obtain the fractions removed by high or low salt buffer from the crude ribosomes, 380 µg ribosomes were incubated on ice for 1 hour in a total volume of 600 µl of either wheat germ buffer (low salt) or 0.5 M KCl buffer (section 2.3.4). The ribosomes were pelleted as described in section 2.3.4 and the supernatants removed. Protein was precipitated by the addition of ammonium sulphate to 70% saturation, redissolved in 100 µl wheat germ buffer and dialysed overnight against 100 ml of the same buffer. In the pre-incubations with the salt washed ribosomes, 50 µl of each protein fraction was included. The same method was used to obtain the low and high salt supernatants from wheat germ ribosomes washed once or twice in wheat germ buffer. In the case of the once washed samples, the crude ribosomes were pelleted, resuspended in the same volume of wheat germ buffer or high salt buffer and incubated as before. For the twice washed samples, the crude ribosomes were pelleted, resuspended in wheat germ buffer, re-pelleted and then resuspended in either wheat germ buffer or high salt buffer. 1. control rRNA not cleaved with aniline; 2. salt washed ribosomes; 3. salt washed ribosomes incubated with 5 µl wheat germ S-100; 4. salt washed ribosomes incubated with the fraction removed by high salt; 5. salt washed ribosomes incubated with the fraction removed by low salt; 6, 9 and 12. crude ribosomes prior to high salt washing; 7. and 8. salt washed ribosomes incubated with the fractions removed from the once washed ribosomes by high salt and low salt, respectively; 10. and 11. salt washed ribosomes incubated with the fractions removed from the twice washed ribosomes by high and low salt, respectively.



1. resuspend in high salt
2. spin
3. resuspend in wheat germ buffer


1 2 3 4 5 6 7 8 9 10 11 12
25S
18S
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5S

comparable to that seen in the ribosomes before they were treated with 0.5 M KCl (track 6). In these experiments 36 ug of ribosomes were used and the amount of the S-100 added to these was such that the stoichiometry was the same as that found in the S-30 i.e. in the wheat germ lysate itself. In the case of the dialysed supernatant from the high salt wash then the amount of protein added back was equivalent to that which was removed from approximately 5 times the amount of ribosomes used in the assay (i.e. 180 ug). It was found that if only the amount of protein equivalent to that removed from the 36 ug of ribosomes in the assay was added in the preincubation then no discernable protection was seen.

The protective effects of these samples is in contrast to the situation when the ribosomes are preincubated with the supernatant from ribosomes washed in the standard low KCl-containing wheat germ buffer (25 mM Hepes/KOH, pH 7.6, 60 mM KCl, 2.5 mM $Mg(OAc)_2$)(Fig. 6.2.1, track 5). In this case there is no reduction in the level of depurination compared to the control incubated without addition (track 2). The amount of protein added back was equivalent to that removed by the wheat germ buffer from 180 ug ribosomes. This was such that it was in the same ratio to the ribosomes as the high salt supernatant. The difference in the ability of this sample to protect salt washed ribosomes from ricin A chain compared to the high salt supernatant suggests that the protection is due to the presence of a factor or factors which are removed by the KCl and which are normally found loosely associated with the ribosomes.

This is borne out further if the ribosomes are washed once or twice by recentrifuging and resuspending in wheat germ buffer prior to

salt-washing in order to remove soluble proteins contaminating the ribosomes. Following the first and second washes the proteins then removed by the high salt are still capable of protecting from ricin A chain (Fig. 6.2.1, tracks 7 and 10). Again this is in contrast to the supernatants from the recentrifuged, low KCl buffer-washed ribosomes which have no protection activity (tracks 8 and 11). Following the second wash in wheat germ buffer, when the ribosomes are then incubated with the dialysed supernatant from the high salt wash of these ribosomes the protection is somewhat reduced, but still significant (track 10).

The proteins contained in the fractions added back were analysed by PAGE and visualised by silver staining as described in section 2.13.1 (Fig. 6.2.2). In the supernatants from the wheat germ buffer-washed ribosomes the total protein is reduced the more times the ribosomes are centrifuged and resuspended. This would be expected since the majority of the proteins are not normally bound to the ribosomes but represent contaminating proteins trapped in the ribosome pellet. However in the supernatants from the KCl washes of these ribosomes, proteins which are only removed by the high salt should be practically unaffected in amount by the prior centrifugations and washings in low salt. This is the case with a 100,000 Da protein (arrowed) which is specifically labelled by diphtheria toxin and ^{14}C-NAD (Fig. 6.2.3) and which is therefore eEF-2. The amount of this protein in the wheat germ buffer-washed fractions is significantly smaller than in the high salt supernatant fractions as measured by the amount of the labelled band on the autoradiograph. There is therefore a correlation between the amount of eEF-2 in the fractions and the

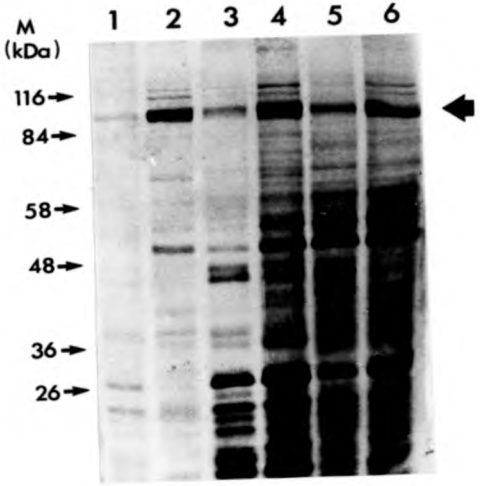
Fig. 6.2.2 Analysis by PAGE of fractions removed by high and low salt from wheat germ ribosomes.

The fractions, assayed for their ability to protect salt washed ribosomes from depurination by ricin A chain in Fig. 6.2.1, were analysed on a 10% SDS polyacrylamide gel and visualised by silver staining (section 2.13.1). 30 µl of each fraction was run on the gel.

1 and 2. Protein removed from the twice washed ribosomes by low and high salt, respectively.

3 and 4. Protein removed from the once washed ribosomes by low and high salt, respectively.

5 and 6. Protein removed from the crude ribosomes by low and high salt, respectively.


M
(kDa)
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6
116
84
58
48
36
26

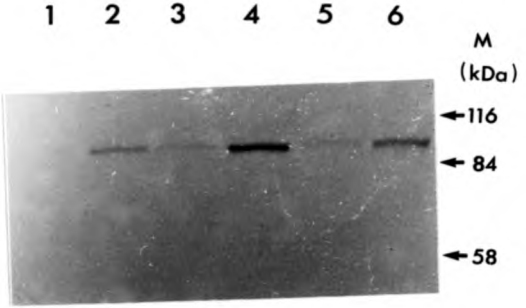
Fig. 6.2.3 Fractions removed from wheat germ ribosomes by high and low salt assayed for eEF-2.

20 µl of each of the samples analysed by PAGE in Fig. 6.2.2 were assayed for eEF-2 as described in section 2.14.6. Following the incubation with diphtheria toxin and ^{14}C NAD the samples were fractionated in a 10% SDS polyacrylamide gel (section 2.13.1) and the ^{14}C labelled eEF-2 visualised by autoradiography.

1 and 2. Fractions removed from the twice washed ribosomes by low and high salt, respectively.

3 and 4. Fractions removed from the once washed ribosomes by low and high salt, respectively.

5 and 6. Fractions removed from the crude ribosomes by low and high salt respectively.


1
2
3
4
5
6
M
(kDa)
116
84
58

ability of these fractions to protect salt-washed ribosomes from depurination by ricin A chain suggesting that it is this protein which is responsible for the protection. This is further substantiated by the fact that in the high salt supernatant from the twice low salt washed ribosomes eEF-2 is the major protein present (Fig. 6.2.2, track 2). The reduced level of protection seen with this fraction (Fig. 6.2.1, track 10) is reflected in the reduced amount of eEF-2 when compared to the other two high salt supernatants (Fig. 6.2.3). This is probably the result of accumulative losses in the amount of ribosomes during the pre-washing in low salt.

6.2.2 Purification of eEF-2 and its Ability to Protect Ribosomes.

In order to confirm that eEF-2 is responsible for the protection, eEF-2 was purified from a wheat germ lysate. Initial attempts to purify the same protein from a yeast lysate proved unsuccessful. The stumbling block was the large amount of starting material which required the growth of 100 litres of yeast culture. The impracticality of this meant that an alternative source was used. The protocol used to purify the protein from wheat germ was an adaptation of the method of Legocki (1979) with the incorporation of the use of a heparin Sepharose column. This has been shown to be highly selective in the purification of eEF-2 from other eukaryotic sources (Giovane et al., 1987). The detailed method is described in section 2.14 and the purification is summarised in Table 6.2.1. The procedure yielded an eEF-2 preparation purifed about 300 fold over the crude postribosomal supernatant. Polyacrylamide gel electrophoresis of the

STEP	TOTAL PROTEIN (mg/ml)	eEF-2 (mg/ml)	FOLD PURIFICATION	YIELD (%)
1. ammonium sulphate precipitation.	40	0.12	1	100
2. DEAE cellulose	22	0.23	3.5	72
3. Phosphate cellulose	11	0.32	10	45
4. Heparin Sepharose	1.5	0.18	40	30
5. Hydroxylapatite	0.25	0.2	267	10

Table 6.2.1 Summary of the purification of eEF-2 from wheat germ.

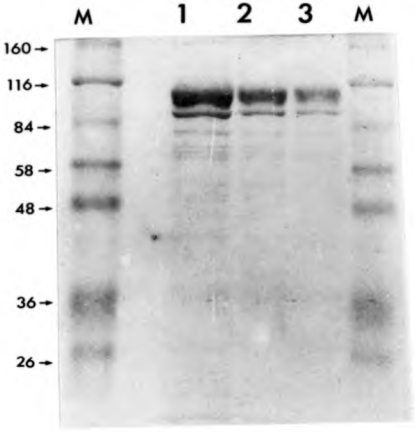
The total protein in each fraction from the purification steps was estimated using the Biorad protein assay with γ-globulin as the standard. The amount of eEF-2 was estimated from triplicate aliquots as described in section 2.14.6.

Fig. 6.2.4 PAGE of purified eEF-2.

To check the purity of the wheat germ eEF-2 aliquots were fractionated in a 10% SDS polyacrylamide gel and stained with Coomassie Blue (section 2.13.1).

Lanes 1, 2 and 3; 10, 4 and 1 µg of protein sample from the final purification of eEF-2, respectively.

M - denotes pre-stained Sigma molecular weight markers.


M
1
2
3
M
160
116
84
58
48
36
26

purified protein showed that it was substantially pure (Fig 6.2.4). A slightly smaller protein present in the purified fraction was also labelled by diphtheria toxin and ^{14}C-NAD and this probably represents a breakdown product formed by proteolysis during the purification. A trypsin sensitive Arg has been found near the N-terminus of a number of eEF-2s (Giovane et al., 1987) and so this smaller protein could be the product of cleavage at this point. In addition this smaller protein is not present in the initial crude homogenate (Fig. 5.3.4). The size of the eEF-2 is larger than the 70,000 reported by Legocki (1979), but from the similarity in the size of eEF-2 from wheat germ and yeast (Fig. 5.2.4), a value of approximately 100,000 Da seems more reasonable. This is because a number of other eukaryotic eEF-2's, including yeast (Skogerson, 1979), have been shown to be proteins of this size (Giovane et al., 1987). Lax et al. (1986) have also reported that wheat germ eEF-2 is a protein of 100,000 Da. The purified eEF-2 was shown to be active in binding to ribosomes using the nucleotide binding assay described by Legocki (1979). In the presence of salt-washed ribosomes, the eEF-2 promoted the binding of labelled GTP (Table 6.2.2).

The ability of the purified eEF-2 to protect KCl-washed ribosomes was tested using the same system previously described (Fig. 6.2.5). When the ribosome were preincubated with just the non-hydrolysable analogue GMPPCP or with GTP, the addition of ricin A chain resulted in a high level of depurination (tracks 2 and 7). The level of depurination was considerably reduced when the ribosomes were preincubated with eEF-2 and GMPPCP (tracks 3 and 4). In contrast little protection was seen when ribosomes were preincubated

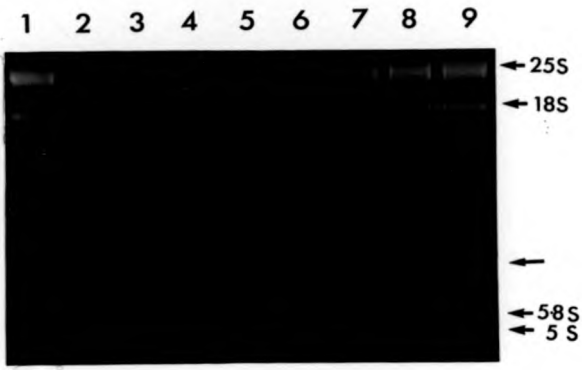
EXPERIMENT	RADIOACTIVITY BOUND (cpm)
1.	109
2.	1149
3.	1069
4.	3955

TABLE 6.2.2 Nucleotide Binding Assay.

Incubations were carried out for 5 min on ice in a final volume of 100 µl containing 40 mM Tris/HCl, pH 7.7, 5 mM $MgOAc_2$, 70 mM KCl, 3 mM DTT and 0.5 µM ^{14}C GTP (450 mCi/mmol). In addition experiment 2 contained 6 µg eEF-2 and experiment 3 contained 18 µg salt washed wheat germ ribosomes. Experiment 4 contained both 6 µg eEF-2 and 18 µg salt washed wheat germ ribosomes. Following the incubation the samples were diluted to 200 µl with ice-cold dilution buffer (10 mM Tris/HCl, pH 7.5, 10 mM $MgOAc_2$ and 50 mM KCl) and filtered through 0.45 µm Millipore HA filters. The filters were washed twice with 300 µl of ice-cold dilution buffer, dried under an ultra-red lamp and counted in 4 ml LKB Optiphase Safe scintillation fluid in an LKB 1212 Minibeta counter.

Fig 6.2.5 Purified eEF-2 assayed for it's ability to protect salt washed ribosomes from depurination by ricin A chain.

36 µg salt washed wheat germ ribosomes were incubated for 10 min at 30 °C with 0.1 µmol GMPPCP (track 2), 0.1 µmol GTP (track 7), 0.1 µmol GMPPCP and 5 µg eEF-2 (tracks 3 and 4), 0.1 µmol GTP and 5 µg eEF-2 (tracks 5 and 6) or with 5 µg eEF-2 (tracks 8 and 9) in a final volume of 100 µl wheat germ buffer. 75 ng ricin A chain was added to each reaction and the incubation continued for a further 15 min. A control without ricin A chain was similarly incubated (track 1). Total RNA was extracted from the reactions, 3 µg treated with aniline and the rRNA fractionated in an agarose/formamide gel as described in sections 2.4.1, 2.5.1 and 2.6.1.


1 2 3 4 5 6 7 8 9
25S
18S
58S
5 S

with eEF-2 and GTP (tracks 5 and 6) or with just eEF-2 (tracks 8 and 9). In these assays the molar ratio of eEF-2 to ribosomes was approximately 2.5:1. The results clearly show that eEF-2 is able to protect ribosomes from the action of ricin A chain and that this protection results from the prebinding of eEF-2 to the ribosomes since this action is only seen under conditions where the factor is prebound irreversibly,i.e. in the presence of GMPPCP. In the presence of GTP and eEF-2 where the factor would be expected to bind in a reversible manner due to the hydrolysis of GTP, a low level of protection is observed. Presumably under these conditions ricin A chain can still compete with the factor for the ribosome.

The conclusion that ricin A chain and eEF-2 compete for the same binding site on the ribosome is substantiated by the observation that the level of protection and the amount of eEF-2 present are directly related (Fig. 6.2.6). As the amount of eEF-2 is increased, the level of depurination is decreased. However, even at very high amounts of the factor there is still a basal level of depurination. Likewise if the amount of eEF-2 is kept constant (25 pmol with 10 pmol ribosomes) and the amount of ricin A chain is increased, then at higher concentrations of the toxin there is less protection (Fig. 6.2.7). Maximal protection is seen when eEF-2 is in greater than a 10 fold molar excess over ricin A chain. But even when the two antagonists are present in equimolar amounts there is still a discernable reduction in depurination (Fig. 6.2.7, track 5) compared to the situation with the toxin on it's own (Fig. 6.2.7, track 4).

The ability of eEF-2 to protect ribosomes from depurination by ricin A chain raised the question of whether other proteins in the

Fig. 6.2.6 Correlation between protection from depurination by ricin A chain and amount of eEF-2.

36 µg salt washed ribosomes were incubated for 10 min with 0.1 µmol GMPPCP (track 2) or with 0.1 µmol GMPPCP and the amounts of eEF-2 indicated in a final volume of 100 µl wheat germ buffer. 75 ng ricin A chain was added and the incubations continued for a further 15 min. A control without ricin A chain was also incubated for 15 min (track 1). Total RNA was extracted, 3 µg treated with aniline and the rRNA fractionated in an agarose/formamide gel as described in sections 2.4.1, 2.5.1 and 2.6.1. The rRNA was stained with EtBr.

1. - ricin A chain control.
2. - eEF-2.
3. 0.2 µg eEF-2.
4. 0.4 µg eEF-2.
5. 0.8 µg eEF-2.
6. 2 µg eEF-2.
7. 4 µg eEF-2.
8. 6 µg eEF-2.
9. 8 µg eEF-2.
10. 10 µg eEF-2.

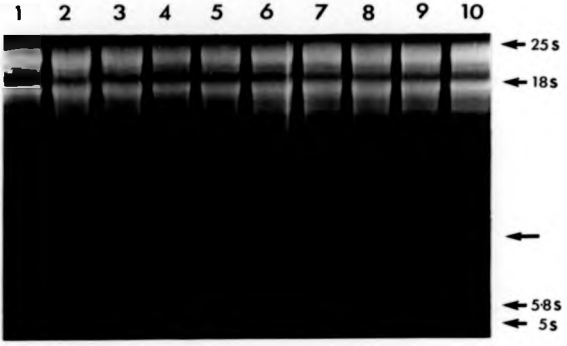
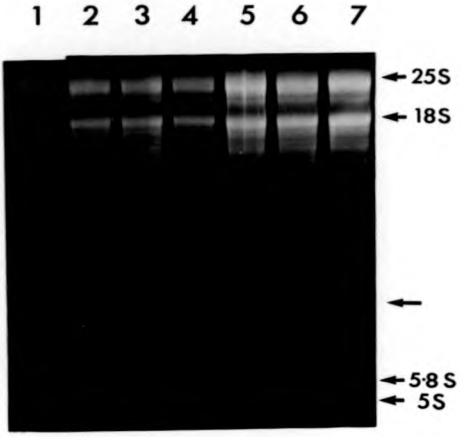

1 2 3 4 5 6 7 8 9 10
25s
18s
5·8s
5s

Fig. 6.2.7 Decreased protection from eEF-2 at higher concentrations of ricin A chain.

10 pmol salt washed ribosomes were incubated for 10 min with 25 pmol eEF-2 and 0.1 µmol GMPPCP or with just 0.1 µmol GMPPCP in a total volume of 100 µl wheat germ buffer. Ricin A chain was added in the amounts indicated below and the incubations continued for a further 15 min. Total RNA was extracted, 3 µg treated with aniline and the rRNA fractionated in an agarose/formamide gel as described in sections 2.4.1, 2.5.1 and 2.6.1.

1. control,- ricin A chain.
2. 250 pmol ricin A chain.
3. 250 pmol ricin A chain and 25 pmol eEF-2.
4. 25 pmol ricin A chain.
5. 25 pmol ricin A chain and 25 pmol eEF-2.
6. 2.5 pmol ricin A chain.
7. 2.5 pmol ricin A chain and 25 pmol eEF-2.


1 2 3 4 5 6 7
25S
18S
5·8 S
5S

postribosomal supernatant were capable of similar protective abilities. Fractions from the different steps of purification of eEF-2 were tested for their ability to protect ribosomes using the same assay described above. From Fig. 6.2.8 it can be seen that only the fractions, which contain eEF-2 and which were subsequently further purified give rise to a decrease in the level of depurination when the ribosomes are preincubated with them. With the exception of fraction DEAE 1, in the other fractions where the eEF-2 has been purified away there is no decrease in the level of depurination when compared to the control, preincubated without addition. DEAE 1 represents the initial flow through from the DEAE-cellulose column and would be expected to contain the bulk of the eEF-1 which unlike eEF-2 does not bind to this matrix (Legocki, 1979). However, there was also a small but significant amount of eEF-2 in this fraction probably due to overloading the column. Attempts were made to purify eEF-1 and whilst this was achieved by approximately 70 fold, the preparation still contained a significant amount of eEF-2. Further purification to remove this resulted in the loss of eEF-1 activity. It was therefore not possible to conclude either way whether or not bound eEF-1 also protected ribosomes from depurination.

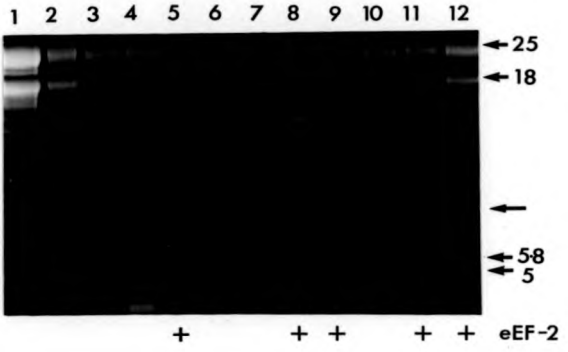
The results presented in this chapter show directly that ribosome bound eEF-2 is able to protect ribosomes from depurination by ricin A chain. When eEF-2 is prebound with the non-hydrolysable analogue GMPPCP, then those ribosomes are protected by a factor of 10-20 fold providing the factor is in excess over the toxin. The results also show that the increase in sensitivity to depurination following treatment of the ribosomes with high salt is most likely due to the

Fig. 6.2.8 The ability of protein fractions from the purification of eEF-2 to protect salt washed ribosomes from ricin A chain.

36 µg salt washed ribosomes were incubated for 10 min with 0.1 µmol GMPPCP and where indicated 15 µl of fractions from the purification of eEF-2 dialysed against wheat germ buffer, in a final volume of 100 µl wheat germ buffer. 75 ng ricin A chain was added to each and the incubations continued for a further 15 min. Total RNA was extracted, 3 µg treated with aniline and the rRNA was fractionated in an agarose/formamide gel as described in sections 2.4.1, 2.5.1 and 2.6.1. The rRNA was stained with EtBr. 1 and 2; controls without ricin A chain. The following tracks were preincubated with the indicated fraction prior to the addition of ricin A chain.

3. No addition.
4. DEAE 1 (flow through from the DEAE cellulose column).
5. DEAE 2 (first peak eluted at 300 mM KCl).
6. DEAE 3 (second peak eluted at 300 mM KCl).
7. P 1 (first peak of unbound protein from the P 11 column).
8. P 2 (second peak of unbound protein from the P 11 column).
9. P 3 (protein eluted with 350 mM KCl).
10. H 1 (unbound protein from the heparin Sepharose column).
11. H 2 (protein eluted with 200 mM KCl).
12 purified eEF-2.

Fractions which contain eEF-2 are marked with a (+).


1 2 3 4 5 6 7 8 9 10 11 12
25
18
5.8
5
+ + + + + + eEF-2

removal of eEF-2 and that the change can be reversed by prior incubation with the same protein.

There have been conflicting reports published on the effect of eEF-2 on the inhibition of protein synthesis catalysed by RIPs other than ricin A chain. Olsnes and Abraham (1979) reported that eEF-2 sensitised rabbit reticulocyte ribosomes to the type 2 RIP modeccin. Although this effect was reduced if GTP was also added, it directly contradicts the effect of eEF-2 on the activity of ricin A chain. Similarly Coleman and Roberts (1981) found that salt-washed rabbit reticulocyte ribosomes were not suitable substrates for inhibition by tritin (the type 1 RIP from wheat germ) and that a factor from the postribosomal supernatant was required for tritin-catalysed inhibition of protein synthesis. It is possible that these conflicting reports have resulted from the use of ribosomes with different conformations and that preincubation with eEF-2 altered them into a form susceptible to RIP attack. This arguement has been proposed by Coleman and Roberts (1981) to explain their earlier results on the requirement for ATP and tRNA in the tritin catalysed inhibition of protein synthesis (Roberts and Stewart, 1979). But it is also possible that these toxins, which have the same site of action, differ in the way they gain access to this site on the ribosome and that this is reflected in the different effects of eEF-2. Unlike ricin A chain, other RIPs may not be inhibited by ribosome bound eEF-2. Indeed the presence of eEF-2 could alter the conformation of their binding sites to make it more accessible.

Sallistio and Stanley (1990) have isolated a number of Chinese hamster ovary cell mutants whose ribosomes show differential

sensitivity to depurination by ricin A chain, abrin A chain and modeccin. In the wild type the ribosomes are equally sensitive to all three toxins, but in some of the mutants the ribosomes were found to be resistant to the action of ricin A chain and abrin A chain, but still sensitive to modeccin. The mutations appear to reflect structural changes in certain ribosomal proteins and the finding that they result in partial resistance to some but not all the RIPs suggests that the accessibility of A_{4324} to each toxin differs. This preliminary investigation would certainly seem to support the notion that there are differences in the action of these toxins, as borne out by the data on the effects of eEF-2 , even though they all share the same site of depurination.

The relationship between the amount of eEF-2 and the activities of these RIPs is touched upon in chapter 8 and the results presented there would seem to throw doubt on the idea that some of the other RIPs differ from ricin A chain in the way their activities are modulated by eEF-2. By using the experiments described above with some of these other RIPs it would be possible to show directly whether in fact there are differences or whether the published results just reflect anomalies in the assays used.

CHAPTER 7.

KINETIC MEASUREMENTS ON THE INACTIVATION OF WHEAT GERM RIBOSOMES BY RICIN A CHAIN.

SECTION 7.1 INTRODUCTION.

In chapter 5 it was shown that wheat germ ribosomes are approximately 1000 times less sensitive to ricin A chain-catalysed depurination than those from rabbit reticulocytes and that this difference is not due to the presence or absence of any supernatant factors. This chapter describes kinetic experiments carried out to investigate the difference in the way ricin A chain depurinates these two substrates.

Kinetic experiments on the action of ricin A chain were first carried out by Olsnes et al. (1975b). They measured the rate of catalysis indirectly by measuring the inhibition of protein synthesis and showed that rabbit reticulocyte ribosomes were inactivated at a rate of 1400 ribosomes/min and that the Michaelis constant (K_m) was 0.2 μM. More recently Endo and Tsurugi (1988) published values of 1777 ribosomes/min and 2.6 μM for the K_{cat} and K_m respectively for the inactivation of rat liver ribosomes. These authors measured the rate of depurination directly by calculating the amount of the ca. 400 nucleotide fragment released by aniline cleavage at the site of depurination.

A prediction of the Michaelis-Menton equation for first order kinetics is that, providing the backward reaction, k_2, is greater than the turnover rate, k_3 , then the K_m becomes a measure of the binding affinity of the enzyme for the substrate. This is substantiated in the case of ricin A chain by the direct measurement of the dissociation constant (K_d). Hedblom et al. (1976) have shown that 1 molecule of ricin A chain binds to one rat liver ribosome with a K_d

of 2 μM. This value is in agreement with the two published values for the K_m of this reaction. Therefore by analogy, values of the K_m and K_{cat} for the ricin A chain-catalysed depurination of wheat germ ribosomes would indicate whether the large difference in sensitivity, compared to rabbit reticulocyte and rat liver ribosomes, was due to a decrease in the binding affinty or the rate of catalysis.

SECTION 7.2 RESULTS AND DISCUSSION.

The rate of catalysis can be measured providing the percentage depurination can be calculated at a given time point and providing the rate is linear over that time period. The aniline assay has been shown to be a good method of measuring the extent of depurination (chapter 3) and it has been used previously to measure the kinetics of ricin A chain catalysed depurination of rat liver ribosomes (Endo and Tsurugi, 1988). These authors used radioactively labelled ribosomes and calculated the amount of the aniline fragment by measuring the radioactive counts and expressing it as a ratio to the 5.8S rRNA in order to calculate the percentage depurination. A similar approach was used with the wheat germ ribosomes, except that the amount of the two fragments was calculated directly from ethidium stained gels. This assumes that in the conditions of the denaturing gel the labelling by ethidium bromide is directly proportional to the length of the RNA fragments.

The lengths of wheat 5S and 5.8S rRNAs are 120 and 164 nucleotides respectively (Mackay et al., 1980, Wildeman and Nazar, 1982). The exact position of the 3' end of wheat 25S rRNA has not

been determined, but by analogy to other LSU-rRNAs Barker *et al.* (1988) have shown that it lies close to position 496 in the sequence for the intergenic region of wheat rDNA. This would make the fragment released by aniline following depurination of the 25S rRNA approximately 366 nucleotides in length.

$$\frac{[\text{ relative amount of the aniline fragment }]}{[\text{ relative amount of the 5.8S rRNA }]} \times \frac{164}{366} \times 100 = \%\ \text{depurination}$$

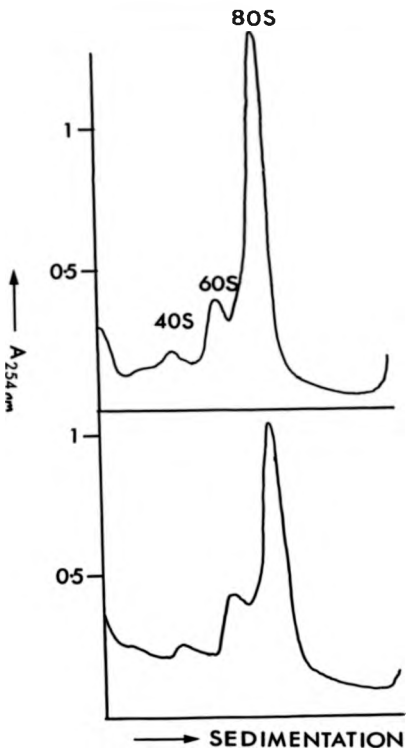
Equation 1.

The relative amount of each fragment was measured by scanning negatives of photographs of rRNA gels with a Molecular Dynamics computing densitometer using the volume integration package. This allows the total absorbance for each band to be calculated and corrected for the background. Equation 1. shows how the percentage depurination was calculated by correcting for the difference in length of the two fragments. This has the advantage that by expressing the fragment relative to the 5.8S rRNA any differences in loadings between the tracks are accounted for because the 5.8S and 25S rRNAs are present in equimolar amounts (Rubin, 1973).

The ribosomes used in the kinetic studies were salt-washed in order to remove the bound elongation factors which might otherwise act as competitors in the assay and distort the measurements. Analysis of the ribosome preparations by sucrose-gradient-density centrifugation showed that the predominant species were 80S monosomes and that there were very few subunits even after KCl treatment (Fig. 7.2.1). Before these measurements were carried out, to test that the

Fig. 7.2.1 Absorbance profiles of crude and salt washed wheat germ ribosomes.

60 µg crude (top) and salt washed (bottom) wheat germ ribosomes were analysed on sucrose gradients as described in section 2.13.2. Following centrifugation the gradients were fractionated and the aborbance across the gradients was measured.


80S
1
0·5
60S
40S
A254 nm
1
0·5
SEDIMENTATION

method of scanning the gels was meaningful, the 5S and 5.8S rRNAs were scanned in 10 tracks and the amounts of each expressed as a ratio. When averaged the mean ratio was 1.42 and the standard deviation +/- 0.1. This is a good approximation to the actual ratio in the lengths of 5.8S rRNA:5S rRNA= 1.37.

The substrate concentrations used for the kinetic measurements were in the range 0.5-2 μM. To find the concentration of ricin A chain needed and the time period over which the reaction is linear, the percentage depurination was measured with different concentrations of ricin A chain over 60 min. Fig. 7.2.2 shows the time course of the reaction when [S]= 1.27 μM and [ricin A]= 0.06 μM. It can be seen that the reaction rate is linear over the first 15 min. The initial rate of depurination was also calculated at lower and higher concentrations of ricin A chain. From Fig. 7.2.3 it is apparent that up to approximately 0.15 μM ricin A chain then the initial rate of depurination is linear with respect to the concentration of ricin A chain. At higher concentrations this is no longer the case. For the following measurements of K_m and K_{cat} a mid-range value of ricin A chain was used i.e. 0.06 μM.

Salt-washed wheat germ ribosomes at concentrations from 0.5 to 1.9 μM were incubated with ricin A chain for 10 min. Samples were removed at 5 and 10 min and the rRNA extracted and treated with aniline. The percentage depurination was calculated at each time point using equation 1 from scans of the rRNA fractionated on agarose/formamide gels. The initial rate of depurination was calculated as an average of the two time points. The data were analysed by a computer-based programme Enzkin 9 which calculated the

Fig. 7.2.2 Time course of the depurination of wheat germ ribosomes.

160 µg salt washed wheat germ ribosomes were incubated at 30 °C for 45 min in a total volume of 30 µl wheat germ buffer with 60 ng ricin A chain. 3 µl aliquots were removed at different time points and the total RNA extracted as described in section 2.4.1. Half of this (4 ug) was treated with aniline and fractionated as described in sections 2.5.1 and 2.6.1. The % depurination was calculated from densitometer scans of the rRNA using equation 1.

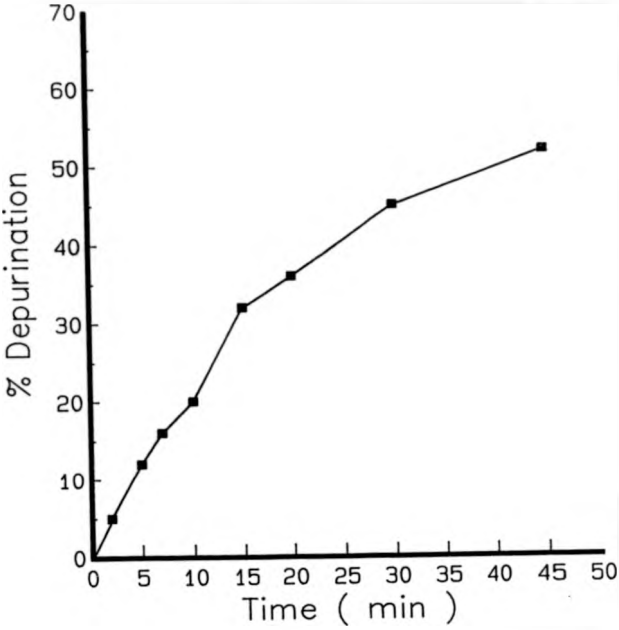
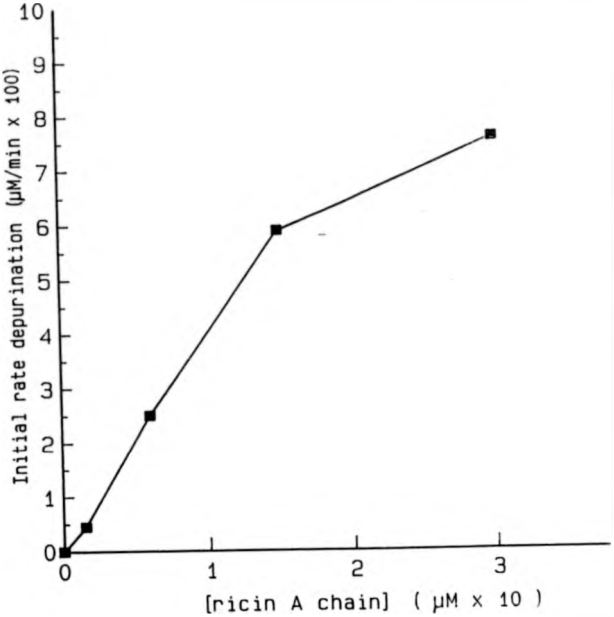

% Depurination
70
60
50
40
30
20
10
0
0 5 10 15 20 25 30 35 40 45 50
Time (min)

Fig. 7.2.3 Relationship between the initial rate of depurination and the concentration of ricin A chain.

160 µg salt washed ribosomes were incubated at 30 °C with a range of ricin A chain concentrations for 10 min in a final volume of 30 µl wheat germ buffer. 3 µl aliquots were removed at 4 and 10 min and the total RNA extracted. 4 µg was treated with aniline and fractionated on an agarose/formamide gel as described in sections 2.5.1 and 2.6.1. The initial rate of depurination was calculated from the % depurination at the two time points assuming that the reaction was linear over this period.


Initial rate depurination (μM/min x 100)
10
9
8
7
6
5
4
3
2
1
0
0
1
2
3
[ricin A chain] (μM x 10)

K_m and V_{max} using a number of reciprocal plots. The detailed methods of calculating the K_m and V_{max} by the different plots are shown in Appendix 2. Of these different methods the Cornish-Bowden is statistically the best because it calculates a median not a mean value for the K_m and V_{max} . Therefore it is almost unaffected by a few aberrant values. Graphs of these plots are shown in Fig. 7.2.4. Similar analysis was carried out on measurements taken in two other similar experiments and the values of K_m and V_{max} calculated from Cornish-Bowden plots of the 3 data sets are shown in table 7.2.1. The average values are 2.1 μM and 0.06 μM/min for the K_m and V_{max} respectively. From the maximum velocity the turnover number or K_{cat} can be calculated at 1.1 ribosomes/min.

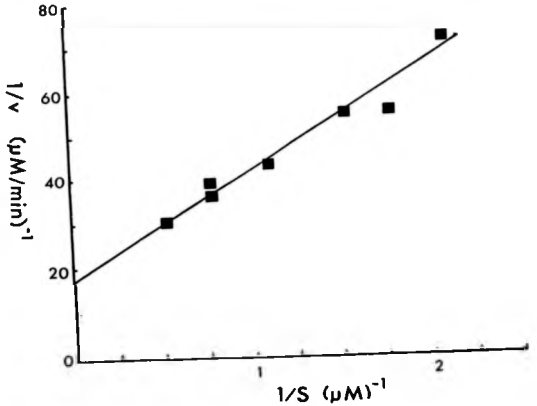
This value of the K_m for the depurination of wheat germ ribosomes by ricin A chain is of the same order of the values reported for rabbit reticulocyte ribosomes (Olsnes et al., 1975b) and rat liver ribosomes (Endo and Tsurugi, 1988). On the other hand the K_{cat} is approximately 3 orders of magnitude smaller. This suggests that ricin A chain is able to bind to all these ribosomes with the same affinity and that the large difference in sensitivity of wheat germ ribosomes compared to the rabbit and rat ribosomes is a reflection of the smaller turnover number. This result explains an earlier observation by Cawley et al. (1977) who found that whilst they did not observe an inhibition of protein synthesis in a wheat germ lysate with ricin A chain, gel-filtration experiments with labelled A chain showed that it bound to the ribosomes in a similar manner to those from rat liver.

Fig. 7.2.4 Reciprocal plots for the reaction of ricin A chain on wheat germ ribosomes.

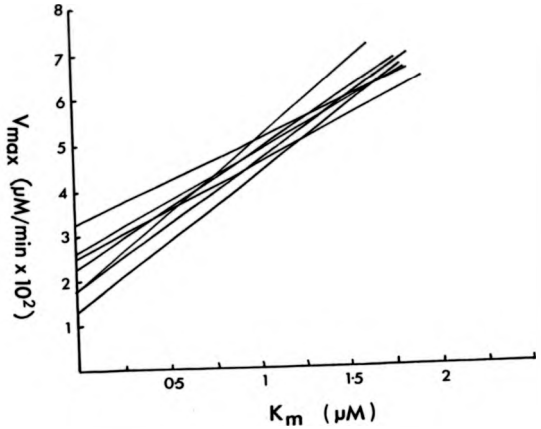
Salt washed ribosomes (60-240 µg) were incubated at 30 °C for 10 min in 30 µl wheat germ buffer with 60 ng ricin A chain. Aliquots (16 µg) were removed at 5 and 10 min and the total RNA extracted as described in section 2.4.1. 4 µg was treated with aniline and fractionated on an agarose/formamide gel as described in sections 2.5.1 and 2.6.1. The gel was scanned and the % depurination calculated at each time point. From this the initial rate of depurination was calculated for each substrate concentration assuming the reactions were linear over this time period.

1. Lineweaver-Burke plot.
2. Cornish-Bowden plot.

1.


80
60
40
20
0
1/v (μM/min)⁻¹
1
2
1/S (μM)⁻¹

2.


8
7
6
5
4
3
2
1
Vmax (μM/min x 10²)
0·5
1
1·5
2
Km (μM)

Km (μM)	Vmax (μM/min)
3.7	0.09
1.34	0.05
1.3	0.054

Table 7.2.1 Measured values for the Km and Vmax for the reaction of ricin A chain on wheat germ ribosomes.

The values were calculated from the Cornish-Bowden plot shown in Fig. 7.2.4 and two other similar experiments.

CHAPTER 8.

COMPARISONS BETWEEN THE ACTIVITIES OF RICIN A CHAIN AND THE TYPE 1 RIPs FROM DIANTHUS CARYOPHYLLUS.

SECTION 8.1 INTRODUCTION.

The leaves of the carnation plant, Dianthus caryophyllus, contain two type 1 RIPs, dianthin 30 and 32. These have been purified to apparent homogeneity and shown to inhibit translation in a rabbit reticulocyte lysate (Stirpe et al., 1981). Dianthin 30 was found not to be present in the initial crude leaf lysate and it was postulated that it might have arisen from dianthin 32 or another protein by proteolysis during the purification. Reisbig and Bruland (1983a) subsequently showed that it was the 60S subunit that was the site of action, in keeping with these proteins being RIPs. Interestingly they found that these proteins were very active in inhibiting translation in a wheat germ lysate and that the ID_{50} for both proteins was in the order of 10 ng/ml. In a reticulocyte lysate 3-5 ng/ml was required to inhibit protein synthesis by 50%. This is in contrast to ricin A chain where the ID_{50} in their system was found to be 1 ng/ml for the reticulocyte lysate, but no inhibition of protein synthesis was found at concentrations of up to 10 µg/ml in a wheat germ lysate. In chapter 5 this large difference in activity on the two different ribosomes was confirmed using the aniline assay and found to be of the order of 1000-fold. In this chapter the N-glycosidase activity of the two dianthins was compared to ricin A chain using the same aniline assay. Kinetic measurements were also carried out in order to investigate the reason for the high activity of the two dianthins against wheat germ ribosomes.

Reisbig and Bruland (1983a) also reported that purified wheat germ ribosomes were 10 times less sensitive to inhibiton of

protein synthesis by the two dianthins than the crude wheat germ lysate. It was concluded that a cofactor might be required for their activity. This is similar to the situation for modeccin (Olsnes and Abraham, 1979) and tritin (Coleman and Roberts, 1981) discussed at the end of chapter 6. With modeccin the factor was found to be eEF-2 and so the effect of this factor on the activity of the dianthins was investigated to see whether it differed from that described in chapter 6 for ricin A chain. Unfortunately a sample of modeccin was not available so it could not be included in these comparisons.

SECTION 8.2 RESULTS AND DISCUSSION.

The preparation of the two dianthins were gifts from Dr F. Stirpe. However analysis of these proteins on polyacrylamide gels was carried out by Dr G. Legname in this laboratory and he found that the two preparations were contaminated with other proteins. Consequently he purified each of the two dianthins using HPLC and it is these preparations which were used in the experiments described below.

Primer extension confirmed that both dianthin 30 and 32 depurinated wheat germ 25S rRNA at the same site as ricin A chain, and that this was the same adenine in a highly conserved sequence shown initially by Endo et al. (1987) to be the sight of action of ricin A chain in rat 28S rRNA (Fig. 3.2.7, chapter 3).

To compare the N-glycosidase activites of the three RIPs 36 µg yeast ribosomes were incubated for 20 min with amounts of toxin ranging from 50 ng to 50 pg. The rRNA was extracted, treated with

aniline and fractionated on agarose/formamide gels. From Fig. 8.2.1 it can be seen that the activities of all three toxins are very similar on yeast ribosomes. There is significant depurination when the ribosomes are incubated with 5 ng or greater than 5 ng of each toxin. In contrast on wheat germ ribosomes both dianthin 30 and 32 are considerably more active than ricin A chain at equivalent concentrations (Fig. 8.2.2). With the latter, as shown in previous chapters, a high amount of ricin A chain (> 500 ng) is required to depurinate wheat germ ribosomes, whereas the dianthins are capable of modifying the ribosomes to the same level of depurination at amounts as low as 5 ng.

The same approach described in chapter 7 was used to measure the K_m and V_{max} for the depurination of salt-washed wheat germ ribosomes by dianthin 32. The same range of substrate concentrations was used and the percentage depurination was measured after an incubation of 4 min with 1 ng of toxin. A second set of data points was obtained by measuring the percentage depurination after 10 min with 0.1 ng toxin. Lineweaver-Burke and Cornish-Bowden plots for both experiments are shown in Fig. 8.2.3 and 8.2.4. The K_m and K_{cat} values calculated from the Cornish-Bowden plots were 1.8 μM and 210 ribosomes/min at the higher concentration of dianthin 32 and 2.4 μM and 620 ribosomes/min respectively at the lower concentration. The differences in these values represent experimental errors. In comparison to the values measured for the same reaction catalysed by ricin A chain the differences in activity on wheat germ ribosomes seems to be due to an increased turnover rate. Both dianthin 32 and ricin A chain have essentially the same binding affinity for wheat

Fig. 8.2.1 Comparison between the activites of ricin A chain and the two dianthins on yeast ribosomes.

36 µg crude ribosomes were incubated for 20 min in a final volume of 100 µl Endo buffer with the amounts of each RIP indicated below and without addition (track -). Total RNA was extracted, 3 µg treated with aniline and the rRNA fractionated on an agarose/formamide gel as described in sections 2.4.1, 2.5.1 and 2.6.1. The rRNA was stained with EtBr.

1, 50 ng; 2, 5 ng; 3, 0.5 ng; 4, 50 pg : ricin A chain.

5, 50 ng; 6, 5 ng; 7, 0.5 ng; 8, 50 pg : dianthin 32.

9, 50 ng; 10, 5 ng; 11, 0.5 ng; 12, 50 pg : dianthin 30.

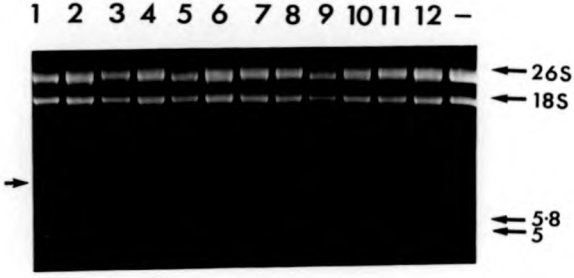

1 2 3 4 5 6 7 8 9 10 11 12 –
26S
18S
5·8
5

Fig. 8.2.2 Comparison of the activities of ricin A chain and the two dianthins on wheat germ ribosomes.

36 µg crude wheat germ ribosomes were incubated for 20 min in a final volume of 100 µl Endo buffer with 5 µg (tracks 1, 5 and 9) , 500 ng (tracks 2, 6 and 10), 50 ng (tracks 3, 7 and 11) or 5 ng (tracks 4, 8 and 12) of each of the three RIPs as indicated below or without addition (track -). The total RNA was extracted, 3 µg treated with aniline and the rRNA fractionated on an agarose/formamide gel as described in sections 2.4.1, 2.5.1 and 2.6.1.

1-4, ricin A chain.

5-8, dianthin 32.

9-12, dianthin 30.

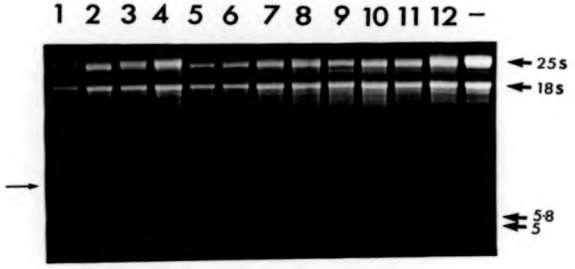

1 2 3 4 5 6 7 8 9 10 11 12 –
25s
18s
5·8
5

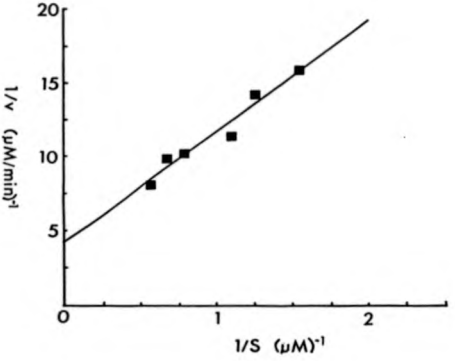
Fig. 8.2.3 Reciprocal plots for the reaction of dianthin 32 on wheat germ ribosomes.

Salt washed ribosomes (80-240 µg) were incubated for 4 min with 1 ng dianthin 32 in a final volume of 30 µl. Total RNA was extracted, treated with aniline and fractionated on an agarose/formamide gel as described in sections 2.4.1, 2.5.1 and 2.6.1. The initial rate of depurination was calculated from densitometer scans of the gel as described in chapter 7.

1. Lineweaver-Burke plot.

2. Cornish-Bowden plot.

1.



2.

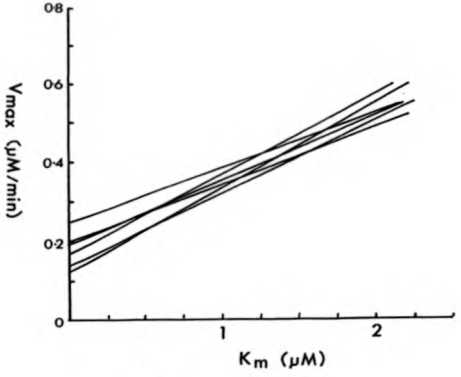
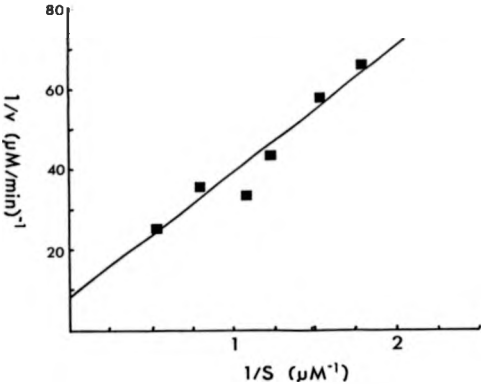


Fig. 8.2.4 Reciprocal plots at a lower concentration of dianthin 32 for the reaction on wheat germ ribosomes.

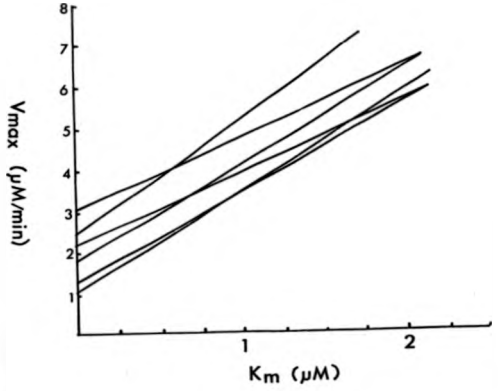
The initial rates of reaction for the depurination of wheat germ rRNA were measured as in Fig. 8.2.3 except that 0.1 ng dianthin 32 was used and the incubation was for 10 min.

1. Lineweaver-Burke plot.
2. Cornish-Bowden plot.

1.



2.



germ ribosomes.

The activity of dianthin 32 was compared on KCl-washed and crude wheat germ ribosomes. In the unwashed ribosomes there is a significant level of depurination when the ribosomes are incubated with equal or greater than 5 ng (Fig. 8.2.5, track 5). Following salt-washing, approximately the same level of depurination is seen at lower levels, 0.5 ng (Fig. 8.2.5, track 9). The difference is argueably less than that seen with ricin A chain, but nevertheless there is an increase in sensitivity. Similarly, this increase is reversed if the washed ribosomes are first incubated with wheat germ eEF-2 and GMPPCP (track 3). Thus, the activity of dianthin 32 on wheat germ ribosomes and the effect of eEF-2 on the activity mirrors that already discussed for ricin A chain. There seems to be no simple explanation why these results contradict those of Reisbig and Bruland (1983a) who found that a cofactor from the wheat germ supernatant was required for activity and that this could be eEF-2. It is possible that the reason why they found that the purified ribosomes were less sensitive than those in the wheat germ lysate was not because of the absence of eEF-2 in the former but because of another cofactor altogether. Even so the high levels of eEF-2 in the wheat germ supernatant might be expected to protect the ribosomes in the wheat germ lysate and result in the wheat germ lysate being less sensitive to the dianthins than the purified wheat germ ribosomes.

Fig. 8.2.5 The effect of salt washing and prior incubation of wheat germ ribosomes with eEF-2 on the activity of dianthin 32.

36 µg crude (tracks 5-7) and salt washed (tracks 8-10) ribosomes were incubated for 20 min in a total volume of 100 µl wheat germ buffer with 5 ng (tracks 5 and 8), 0.5 ng (tracks 6 and 9) or 50 pg (tracks 7 and 10) dianthin 32 and 0.1 µmol GMPPCP or with just GMPPCP (track 1). Alternatively the salt washed ribosomes were preincubated for 10 min with 5 µg eEF-2 and 0.1 µmol GMPPCP prior to the addition of 5 ng (track 2), 0.5 ng (track 3) or 50 pg (track 4) dianthin 32. Following the 20 min incubation total RNA was extracted and 3 µg treated with aniline (sections 2.4.1 and 2.5.1). The rRNA was fractionated on an agarose/formamide gel and stained with EtBr as described in section 2.6.1.


1 2 3 4 5 6 7 8 9 10
25S
18S
5·8
5

CHAPTER 9.

DISCUSSION

Prior to 1987 research into the action of ricin A chain and related plant RIPs had given rise to a bewildering set of results which were often contradictory and which allowed no overall consensus to be reached concerning their mode of action or the consequences of that action on the activity of their substrate, the eukaryotic ribosome. The main stumbling block seems to have been the problem of only being able to measure the activity of these proteins by their inhibitory effect on protein synthesis. Without a direct assay for their activity it was not possible to be certain that the results obtained under different conditions were a consequence of this activity or merely reflected the different levels of this activity under the different conditions. In particular it could not be shown that the degree of modification catalysed by these proteins was the same when the results from different assays were compared. The reviews published on these results (Olsnes and Pihl, 1982, Vazquez, 1979) highlight the conflicting data and the conclusions drawn by different research groups.

The picture was somewhat clarified by the publication of a series of papers which showed that ricin A was a highly specific N-glycosidase (Endo et al., 1987, Endo and Tsurugi, 1987, Endo and Tsurugi, 1988). In particular the aniline assay described by these authors meant that the activity of ricin A chain could to be measured directly for the first time, independently of the subsequent inhibition of protein synthesis. The main theme of the experiments described in the previous chapters has therefore been to readdress some of the aspects of the action of ricin A chain using the aniline assay as the measure of activity. This has had the consequence that

it was possible to avoid some of the ambiguities which were present in many of the previous experiments published prior to the elucidation of the mode of action of ricin A chain. It also allowed for experiments to be done under conditions not dictated by requirements for a protein synthesising system and not complicated by the need to do the experiments under conditions where the concentrations of cations and supernatant factors were not similar to the *in vivo* environment.

The initial localisation of the site of depurination in a sequence in rat 28S rRNA which is conserved in all the LSU-rRNAs investigated to date suggested that the site of action was the same in all susceptible ribosomes. The results of primer extension analysis substantiated this. In all three different types of ribosomes tested, including the plant ribosomes, the site of depurination was found to be at the same adenine in this sequence. The two type 1 RIPs, dianthin 30 and 32, were also found to act at the same point in the 25S rRNA of wheat germ. Indeed the increasing number of reports in the literature all show that these related proteins have the same site of action as ricin A chain and that all the susceptible ribosomes tested to date are modified at the same position.

The similarity in the action of ricin A chain on the different substrates raises the question of what determines the way it recognises these substrates and the factors which affect this. It was shown directly that eEF-2 is able to protect wheat germ ribosomes from depurination by ricin A chain and that this protection is only apparent under conditions where the factor is strongly bound to the

ribosome. The conclusion from this that the two proteins share mutually exclusive binding sites on the ribosome substantiates earlier work into the effects of added eEF-2 on the ricin A chain catalysed inhibition of protein synthesis. In these papers the authors also found that the added factor reduced the amount of inhibition, although they were not able to conclude whether this was due to a reduction in the level of modification or a reversal of activity of modified ribosomes (Fernandez-Puentes et al., 1976a and 1976b, Olsnes et al., 1975b). The results presented here support the first explanation. This is also supported by the observation that prebound eEF-2 reduces the amount of labelled ricin A chain that binds to the ribosome (Cawley et al., 1979).

The finding that this ability to protect ribosomes was also apparent in a fraction that contained eEF-1 raised the question of whether this protein can also similarly protect ribosomes. Unfortunately because of contaminating eEF-2 in the partially purified fraction it was not possible to say anything conclusive on this. There have been conflicting reports in the literature, with some reports that eEF-1 does reduce the level of inhibition (Fernandez-Puentes et al., 1976a) and others showing no affect (Fernandez-Puentes et al., 1976b, Olsnes et al., 1975b). The two elongation factors have been shown to have mutually exclusive binding sites on the ribosome (Richter , 1972) and both have been reported to interact directly with the ricin A chain/α-sarcin loop in E.coli ribosomes (Moazed et al., 1988). It would therefore be plausible that the ribosomal binding sites of both elongation factors could overlap with the binding site of ricin A chain. Indeed it seems

unlikely that ricin A chain and eEF-1 can interact with bases on the rRNA which are identical and not be sterically hindered by each other. The reported instability of purified eEF-1 (Golinska and Legocki, 1973) and the observation that eEF-1 binds only weakly to ribosomes even in the presence of aminoacyl-tRNA and GTP (Nolan et al., 1975) might explain the differences in the previous reports.

Whilst the eEF-2 content of ribosomes affects the sensitivity of different ribosomes to depurination by ricin A chain there are still large inherent differences in the ability of different ribosomes to act as a substrate for the reaction. Wheat germ ribosomes were found to be in the order of 1000 times less sensitive than those from rabbit reticulocytes. Furthermore kinetic studies showed that the difference is due to a reduction in turnover number for the reaction on wheat germ ribosomes. Ricin A chain binds to the two different ribosomes with essentially the same affinty. Endo and Tsurugi (1988) have reported that ricin A chain is able to depurinate naked 28S rRNA and that the K_m for this reaction is the same as for the intact rat ribosome. They have suggested that ricin A chain recognises a specific structure in the rRNA and that the recognition does not require ribosomal proteins. This is supported by the observation that ricin A chain retains its specificty when the substrate is a naturally occuring fragment of 553 nucleotides derived from the 3' end of 28S rRNA (Endo and Tsurugi, 1988) and when a synthetic oligoribonucleotide which mimics the conserved loop around the site of action is used as the substrate (Endo et al., 1988a). If ricin A chain is just interacting with the rRNA in this region then this might explain the similarity in binding affinites for the

rabbit and wheat germ ribosomes, since in both cases the sequence and presumably the secondary structure is highly conserved between the two.

The assumption, based on an increasing number of reports that all plant RIPs depurinate the LSU-rRNA at the identical position raises another question. Namely as to whether all these proteins gain access to the site of action in the same way. The comparisons between ricin A chain and the two type 1 RIPs dianthin 30 and 32 showed that whilst all three proteins had similar activities against yeast ribosomes the two dianthins were in the order of 100 times more active against wheat germ ribosomes. However this difference does not appear to be due to differences in the binding of the three proteins since the K_m for the reaction on wheat germ ribosomes was found to be similar for ricin A chain and dianthin 32. Again this would suggest that the different RIPs are interacting with the same site on the ribosome and that the differences in activities is a reflection of the ability of the various proteins to then hydrolyse the glycosidic bond in the rRNA once they have bound. This is also bourne out by studies on the kinetics of the reaction of PAP on rat liver ribosomes where the K_m was reported to be 1 μM and the K_{cat} 400 ribosomes/min (Ready et al., 1983). In all cases therefore when the kinetics of the inactivation of different ribosomes by different RIPs has been studied, it has been found that they interact with the substrates with apparently the same affinity. The large differences in the rates of the overall reactions seems to be a consequence of the varying abilities of the RIPs to catalyse the reaction after binding. Endo and Tsurugi (1988) have suggested that it is at this second step,

after binding, that the ribosomal proteins play a part in conditioning the action of ricin A chain. This is because their removal in the naked 28S rRNA did not alter the K_m relative to the intact ribosome but did drastically lower the K_{cat}.

There are however a number of reports in the literature which would appear to suggest that the assumption that all these proteins depurinate the rRNA at the same nucleotide by first binding to the same site is an over simplification. The type 1 RIP MAP (Mirabilis antiviral protein) is equally active in a wheat germ lysate and a rabbit reticulocyte lysate but more interestingly it inhibits translation in an E.coli cell-free translation system (Habuka et al., 1990). The IC_{50} concentration was found to be only 100 times greater for the E.coli translation system than the rabbit reticulocyte system. Furthermore it has been shown that the E.coli ribosomes are modified at the same site in the conserved loop i.e. A_{2661} (Noriyuki Habuka, personnal communication). In addition it has been shown using the aniline assay in this laboratory by Martin Hartley that PAP (Pokeweed antiviral protein) also modifies E.coli 23S rRNA. The activity of these RIPs is in contrast to most other plant RIPs including ricin A chain which are inactive against bacterial ribosomes.

Ricin A chain has been shown directly not to bind to E.coli ribosomes (Hedblom et al., 1976) although naked E.coli 23S rRNA does act as a substrate for the reaction (Endo and Tsurugi, 1988). The K_m and K_{cat} for this reaction were almost the same as for naked 28S rRNA supporting the assumption discussed above that ricin A chain is recognising a specific structure in the RNA. It seems therefore

that ricin A chain is prevented from binding to E.coli ribosomes by interference from ribosomal proteins. The finding that this is clearly not the case with some of the other RIPs would indicate that they differ in the way or the place to which they bind on the ribosome.

There is also some evidence in the literature on the effect of eEF-2 on the activity of RIPs which raises questions about possible differences in the way they interact with the ribosome. The experiments described in chapter 6 directly show that the binding of ricin A chain and eEF-2 is mutually exclusive and that a part of their binding sites must overlap. This was also shown to be the case for the type 1 RIPs dianthin 30 and 32 contradicting earlier reports which suggested that their activity might be enhanced by a cofactor and that this could be eEF-2. However in the light of other reports on the activity of modeccin and the stimulation found when eEF-2 was added (Olsnes and Abraham, 1979) it would perhaps be unwise to dismiss this and conclude that all plant RIPs bind in a manner dependant on eEF-2 not being bound first. If a sample of modeccin had been available it would have been interesting to repeat many of the experiments with eEF-2 and then a more comprehensive picture could have been drawn.

The second part to the over all question of the action of ricin A chain tackled in this thesis is the question of how the activity of the ribosome is affected once the rRNA has been depurinated. Although it has not been directly shown that the removal of the single adenine is sufficient to cause the subsequent inhibition of protein synthesis there is no evidence that these

proteins have any other activity on the ribosome. Without this it is probably safe but perhaps not scientifically correct to assume that it is solely the removal of this one base which results in the inhibition of ribosome activity.

By using an assay which allowed effects on initiation and elongation to be distinguished it was possible to show that ricin A chain does not act soley on the elongation cycle as previously thought. In a rabbit reticulocyte lysate ricin A chain was found to reduce the rate of initiation by approximately six-fold relative to the rate of initiation in a lysate inhibited by an inhibitor which acts solely on elongation. Since ricin A chain has been shown to act solely on the 60S subunit and not on any other component of the translational apparatus it must be the formation of the 80S initiation complex from the 48S preinitiation complex which is inhibited. This final step in the initiation pathway is catalysed by eIF-5 and is accompanied by the hydrolysis of GTP and the release of a number of other initiation factors which were part of the 48S preinitiation complex. Little is known about the binding sites of the various initiation factors on the ribosomal subunits but it is possible that the ricin A chain-catalysed inhibition of initiation is the result of changes in the binding site(s) of one of these proteins. This could possibly be the result of the conformational changes induced by the modification (Paleologue et al., 1986). Alternatively the GTPase activity associated with the joining of the 60S subunit might be inhibited by the ricin A chain-catalysed modification of the 60S subunits. This hydrolysis has been shown to be necessary for the final step of initiation to take place (Trachel

et al., 1977) and ricin A chain has been found to affect the GTPase activity of the ribosome associated with the elongation cycle (Benson et al., 1975, Sperti et al., 1975).

Whilst it is clear from these experiments that both initiation and elongation are affected, protein synthesis is shut down on ricin A chain modified ribosomes because elongation is severely inhibited. Dipeptide analysis showed that the majority of the newly initiated but modified ribosomes were only capable of completing at most one round of elongation. The analysis of the peptides formed showed that the predominant species formed was the met-val dipeptide suggesting that it was the eEF-2 catalysed step of elongation which was inhibited by ricin A chain. This was supported by the similarity in the peptides formed in a diphtheria toxin inhibited lysate which is known to only inhibit the translocation step of elongation. Furthermore inhibition by anisomycin which inhibits the peptidyl transferase activity of the ribosome gave an entirely different pattern of methionine-containing peptides. This kind of analysis does however have the disadvantage that the inhibition may be leaky and so additional peptides may be formed from inhibition after one or more rounds of elongation which would complicate the picture. This was found to be the case with anisomycin where in theory only radioactive met should be seen but in practise a number of met containing peptides were formed. To a lesser extent this was probably also the case with the ricin A chain and diphtheria toxin inhibited lysates where the dipeptide was predominant but two other peptides of higher R_f were also formed.

The conclusion that modificaiton of the rRNA by ricin A chain

results in inhibition of translocation was further supported by the finding that the peptides formed in the inhibited reticulocyte lysate and also in an inhibited yeast lysate were unreactive with puromycin. The suggestion from these experiments being that inhibition of translocation would result in the peptidyl-tRNA occupying the ribosomal A site which would then be blocked for the binding of puromycin. Therefore all three experiments carried out to investigate the step of elongation which is inhibited by ricin A chain point towards the eEF-2 catalysed step of translocation. There is no evidence that the eEF-1 catalysed binding of aminoacyl-tRNA is the primary step to be inhibited although it could be argued that the results would be consistent with a partial inhibition of this step. So whilst it would be possible to reconcile these results with earlier reports that found that both of the elongation factor catalysed steps were inhibited by ricin A chain they are at odds with reports which show only an effect on the eEF-1 catalysed step (Endo et al., 1988d, Igarashi et al., 1987). Although these were carried out using the related bacterial RIPs if all these proteins have the same activity then the resultant inhibition must be the same. There would appear to be no obvious reason which would explain this contradiction other than to reiterate what was said in previous chapters. Namely that the experiments described here were carried out by using cell free translation systems where the conditions are closer to the in vivo environment and not by using a poly (U) directed assay on salt-washed ribosomes. This second system used by other authors relies on the addition of large amounts of purified elongation factors and on the concentrations of cations, in

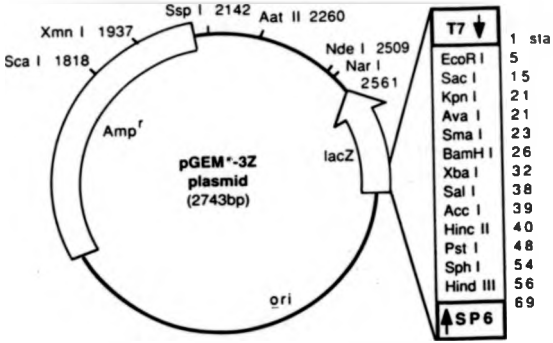
particular Mg^{++}, to be high. Both of which have been shown either in this thesis or in other papers to affect the activity of ricin A chain.

Not withstanding these arguements, it is clear that elongation is severely inhibited in ricin A chain-modified ribosomes. Presumably, however, the importance of this to the overall inhibition of protein synthesis *in vivo* relative to the additional effect on initiation would vary in a manner dependent on the overall rate of protein synthesis. In a system dependant primarily on initiation then the inhibition of initiation by ricin A chain might be expected to contribute more significantly to the final shut down of protein synthesis. On the whole the finding that modified ribosomes are only capable of completing one or two rounds of the elongation cycle would suggest that this is the primary effect of ricin A chain on protein synthesis and that the reduction in the rate of initiation is of secondary importance.

Now that the overall picture of the way ricin A chain interacts with and inactivates ribosomes *in vitro* is becoming clearer, it is perhaps surprising that this has yet to shed any light on its role in the plant, or indeed on the role of any of the plant RIPs. Whilst there is clear evidence that these proteins are anti-viral agents and that this probably results from the inactivation of the host ribosomes through their N-glycosidase activity, the reported resistance of the ribosomes from many plants to their endogenous RIP suggests that this cannot be their function *in vivo*. However it has yet to be directly shown using the aniline assay that this is in fact the case. It is possible that the ambiguities present in some of the

literature in this field also apply here and that a detailed investigation of the ability of a range of RIPs to depurinate the ribosomes from the plants in which they are expressed, as measured by the aniline assay, would in fact show that they are susceptible. Unfortunately, time or the lack of it meant that this was outside the scope of this thesis.

APPENDIX.



Appendix 1. Plasmid map of pGEM-3Z.

The plasmid was obtained from Promega.

Appendix 2. Kinetic Equations.

From the Michaelis-Menton equation, $$v = \frac{Vmax \times [S]}{Km + [S]}$$

the double-reciprocal or Lineweaver-Burke plot can be derived :

$$1/v = (Km/Vmax) \times 1/[S] + 1/Vmax$$

When 1/v is plotted against 1/[S] then the values for the Km and Vmax can be calculated from the intercept with the Y axis and the gradient. Alternatively, the equation can be rearranged to form the Cornish-Bowden plot :

$$Vmax/v - Km/s = 1$$

This has the form $y/b - x/a = 1$ which is the equation of a straight line in xy space where b is the intercept of the line on the y-axis and a is the intercept on the x-axis. Any single observation of v and [S] can be represented by a line passing through v on the Vmax axis and -[S] on the Km axis. If such a line is drawn then the common intercept has the coordinates Km and Vmax. In practise the observations are subject to error and a cluster of intersections will be obtained. The values of Km and Vmax are then taken from the median when the intersections are ranked in order.

STRAIN		GENOTYPE
1. Yeast : (*S. cerevisiae*)	ABYS 1	*a*, *pra* 1, *prb* 1, *prc* 1, *cps* 1, *ade*
2. *E.coli* :	GM 119	*dcm*, *dam*
	TG 1	K12, (*lac*–*pro*) *sup* E, *thi*, *hsd* D5 [F' *tra* D36, *pro* A^+B^+, *lac* I^q, *lac* Z M15]

APPENDIX 3. Yeast and bacterial strains used in this study.

The origin of each strain is referenced in the text.

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